Using Algae as Environmental Impact Indicators in Urban Freshwater Ponds

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2003

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A Thesis submitted in partial fulfilment
of the requirements of the
University of Abertay Dundee
for the degree of Doctor of Philosophy

December 2003

I certify that this thesis is the true and accurate version of the thesis approved by the examiners.

Signed... [redacted] ..... Date..22/12/2003
Director o

Declaration

I hereby declare that the following thesis is based on the results of investigations conducted by myself, and that this thesis is of my own composition. This thesis has not, in whole or part, been previously presented for a higher degree. Work other than my own is clearly indicated in the text by reference to the relevant researchers or their publications.

Signed....  .....

Claire Johnstone

Acknowledgements

I would like to express my sincere gratitude to my supervisor Dr Erica Benson for her guidance, support, continued encouragement, enthusiasm and kind words throughout this project. In addition, I would like to thank Dr John Day and Professor Bill Block for their algal and plant ecology expertise, Dr Chris Jeffries for supplying sampling equipment and the study sites and Dr Kate Heal for sediment data.

My thanks also to Professor Harry Staines for his guidance and support with the wonderful world of statistics!!!

Thanks also to my dear crazy German friend Adolf, without whom my sampling days to Dunfermline would have been much less eventful and hilarious!!!

Jo, Audrey, and Sarah thanks for the much needed distractions and wild nights out.

And finally but by no means least, a big thank you to my mum, dad and sister for keeping me sane and mopping up the tears. Without you guys I would never have made it through.

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Abbreviations

A	
Absorbance at X nm	A_x
2,2' –azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid)	ABTS
Ammoniacal nitrogen	NH_3-N
Analysis of variance	ANOVA
B	
Bovine serum albumin	BSA
C	
1-chloro-2,4-dinitrobenzene	CDNB
Chlorophyll *a*	Chl. *a*
Chlorophyll *b*	Chl. *b*
Culture Collection of Algae and Protozoa	CCAP
D	
Degrees Celsius	°C
Degrees of freedom	D.F.
Deionised water	dH_2O
Dunfermline Eastern Expansion	DEX
5,5′-dithiobis-(2-nitrobenzoic acid)	DTNB
Dimethylsulphoxide	DMSO
E	
Environment Agency	EA
Electron paramagnetic resonance	EPR
Ethylenediaminetetraacetic acid	EDTA
Euglena gracilis medium	EG
F	
Fluorescein diacetate	FDA

G

Gas chromatography	GC
Glutathione reductase	GR
Glutathione-s-transferase	GST
Gram	g

H

Hour	hr
Hydrochloric acid	HCl
Hydrogen peroxide	H_2O_2
Hydroxyl free radical	$^{\cdot}OH$

I, J

Jaworski's Medium	JM

K, L

Lauryl sulphate (sodium dodecyl sulphate)	SDS
Liquid nitrogen	LN

M

Magnesium carbonate	$MgCO_3$
Methane	CH_4
Methyl radical	$^{\cdot}CH_3$
Microgram	µg
Microlitre	µl
Miligrams per litre	$mg.l^{-1}$
Milligrams per millilitre	$mg.ml^{-1}$
Millilitre	ml
Millimolar	mM
Minute	min

N

Nanometer	nm
Nicotinamide-adenine dinucleotide phosphate	NADP

Nicotinamide-adenine dinucleotide phosphate (reduced NADP)	NADPH
Nitrate	NO_3^-
Nitrite	NO_2^-
Nitrotetrazolium blue	NBT
Not available	no sample
Not significant	n/s

O

Oxidised glutathione	GSSG

P

Parts per million	ppm
Percentage	%
Peroxide ion	O_2^{2-}
Phosphate buffered saline	PBS

Q, R

Reactive oxygen species	ROS
Reduced glutathione	GSH
Revolutions per minute	rpm

S

Scottish Environment Protection Agency	SEPA
Second	sec
Singlet oxygen	$^1O_2$
Sodium hydroxide	NaOH
Species	spp.
Sulfhydryl groups	SH
Superoxide dismutase	SOD
Superoxide radical	$O_2^{\cdot-}$
Suspended solids	SS
Sustainable Urban Drainage Systems	SUDS

T

Total oxidised nitrogen	TON
Trichloroacetic acid	TCA
Triplet oxygen	$^3O_2$
Tris-hydrochloride	Tris-HCl

U

Ultraviolet	UV

V

Volume/volume	v/v

W

Weight/volume	w/v

X,Y,Z

Abstract

Using Algae As Environmental Impact Indicators In Urban Freshwater Ponds

Claire Johnstone

Urban freshwater ponds are bodies of water that sustain a diversity of higher plants, vertebrates, invertebrates, amphibians and algae. The importance of ponds in the enhancement of diversity in urban habitats is little understood due to a lack of available knowledge of the processes and interactions that operate within the biotic components of these small aquatic ecosystems. The objectives of this study were to explore the use of algae as indicators of the biotic and abiotic challenges that occur in Sustainable Urban Drainage Systems (SUDS) and to investigate the relationships between algal population dynamics, and stress physiology and water chemistry and quality. An output of this study is the recommendation of management strategies which support the sustainable enrichment of diversity in SUDS. A longer-term consideration is to explore the potential for developing algae as potential phytoremediators in SUDS ponds.

Three ponds were studied in Duloch Park, Dunfermline, Fife. Water quality and algal distribution studies indicated that excessive nutrient loads from surface water runoff, caused the ponds to become eutrophic during most of the course of the study. The ponds were particularly impacted by periodic inputs of suspended solids, road salts, and runoff from construction sites and soil erosion incidents. *Cladophora glomerata* (filamentous blanket weed) was the major algal species present in the ponds and was thus selected as the key indicator organism throughout the study. Due to the short pond retention times, planktonic microalgal populations were extremely low despite adequate nutrient supplies being available. Chlorophyll *a/b* ratios for *C. glomerata* fell below the normal chlorophyll ratio of 2.6:1 indicating excessive exposure to high irradiance. A relationship may also exist between the decline in *C. glomerata* abundance and heavy metal accumulation in the sediments, competition effects due to excessive plant growth of *Phragmites australis* and duckweed and pond turbidity.

At the biochemical level the investigation of a total antioxidant assay (based on the chemical reagent ABTS) was developed for *C. glomerata* to assess sub-cellular stress responses as markers of environmental change. Fluctuations in total antioxidant activity were assigned to combinations of biotic and abiotic stress, life cycle changes and turbidity impacts within the ponds. More detailed studies of individual antioxidants (superoxide dismutase, catalase, peroxidase, glutathione reductase and glutathione-s-transferase) demonstrated that C. *glomerata* had increased SOD activities. Enzymes associated with the removal of toxic H_2O_2 were detected in samples from specific locations and time frames. Depletion in protective non-protein and protein bound SH groups in certain pond samples indicated a significant level of oxidative stress possibly caused by xenobiotics. High levels of glutathione reductase activity were detected in all *C. glomerata* samples suggesting efficient enzyme recycling. It is proposed that this helps protect algal cell membranes from further oxidative damage.

As sampling of algae from ponds is restricted to seasonal growth patterns and availability of algal mats an *in vitro* assay was devised to detect oxidative markers of stress ($^{\cdot}OH$) *in vitro.* This was developed using the microalga *Euglena gracilis* and comprised a non-destructive gas chromatographic technique. Experiments were

constructed to simulate xenobiotic stresses *in vivo*. Cells exposed to high iron and salt concentrations at levels similar to those detected in the ponds, demonstrated high antioxidant activities, suggesting that algae and specifically *E. gracilis* may be a suitable candidate for phytoremediation programmes in SUDS.

The study concludes by presenting an integrated scheme demonstrating the importance of understanding SUDS biotic components at environmental, physiological and biochemical levels. Using this scheme recommendations for improving pond management strategies are made with a view to maximising the biological potential, diversity and sustainability of SUDS ponds in the Scottish urban landscape.

Chapter 1 INTRODUCTION

1 Aims of the project

This thesis explores the use of algae as an indicator organism for the biotic and abiotic challenges that occur in urban freshwater ponds. The research will combine the study of algal population dynamics, environmental biochemistry (photosynthetic pigments, oxidative stress physiology); and water chemistry/quality and hydrology, and seeks to recommend management strategies which support sustained habitat enrichment in urban water systems.

Specific aims include:

- The assembly of data on aquatic environmental parameters and to construct comparative profiles of algal diversity, distribution and population dynamics of selected urban ponds;
- The critical evaluation of the usefulness and interpretation of pond survey questionnaire data for a SUDS pond monitoring programme;
- The development and application of a new total antioxidant assay for the study of total antioxidant enzyme activity in macroalgae collected from urban ponds;
- The critical evaluation of the novel use of algae as environmental impact indicators in urban ponds using knowledge of oxidative stress physiology linked to stress challenges (e.g. pollution);
- An in depth study of oxidative stress and antioxidant defence in macroalgae with a view to investigating the role of algae as potential phytoremediators in urban ponds;

- Utilising knowledge derived from the study to develop criteria for the improved management of SUDS' in a biological context.

1.1 Introduction

Ponds in the urban landscape offer a unique biodiversity resource and are generally a visual focus in the landscape providing an amenity for local communities. Ponds are unique bodies of water that may sustain a variety of freshwater plants, invertebrates, amphibian and algal species and they are also important habitats for many birds and animals. However, the importance of ponds in creating habitats of diversity can be overlooked not only by the general public but also by environmental bodies, due to a lack of available knowledge of the processes and interactions that operate within small aquatic ecosystems. It is anticipated that by acquiring a greater understanding of how small aquatic ecosystems function, it will be possible to recommend management strategies that provide the potential to sustain habitat enrichment in urban water systems (Day *et al.*, 1999). Consequently, the inspiration for this project is based on the consideration of Sustainable Urban Drainage Systems (SUDS) in a holistic sense, to enrich the biodiversity of the urban landscape and to offer practical management strategies for SUDS operations.

1.2 Sustainable development and urban drainage

Sustainable development was the central theme of the UN Earth Summit at Rio de Janeiro in 1992, which required governments to produce their own sustainability strategies. Sustainable urban drainage is a concept that includes long term environmental and social factors in decisions regarding drainage. It takes account of the quantity and quality of runoff and the amenity value of surface water in the urban

environment (www.ciria.org.uk/suds.html). Many existing urban drainage systems can cause problems of flooding, pollution or damage to the environment and are not proving to be sustainable.

Increasing urbanization through the development of buildings, roads and other impermeable surfaces result in alterations to the natural hydrological cycle. Traditionally in the UK, new urban sites have been engineered in such a way that surface water is drained as quickly as possible to the nearest watercourse to prevent the possibility of flood occurrence (Revitt *et al.*, 1999). The design of such a system however, neglects the potential pollutant loads that can be generated in urban runoff, particularly after a prolonged dry period which can have significant impacts on receiving water quality (D'Arcy, 1998). The pollutant load tends to be highly variable due to a dependence on factors such as land use, catchment area, frequency of storms and weather conditions between storms. The principal pollutants in urban runoff are suspended solids, heavy metals, nutrients, hydrocarbons and faecal coliforms from foul to storm cross connections (Horner *et al.*, 1994; D'Arcy, 1998).

1.2.1 Sustainable Urban Drainage

Sustainable Urban Drainage is primarily concerned with drainage of rainwater from developed or urbanised areas and takes into account water quantity, water quality and amenity issues (Working SUDS Party, 1999).

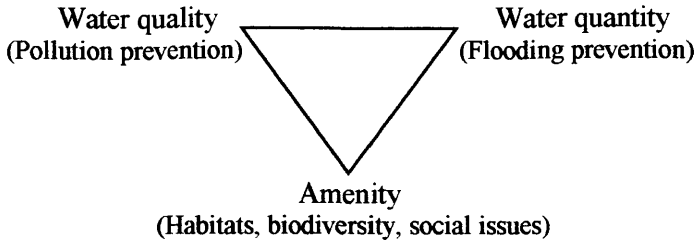


Figure 1.1 The Urban Drainage Triangle (Working SUDS Party, 1999)

These systems are more sustainable than conventional drainage methods because they: a) manage runoff flowrates by reducing the impact of urbanisation on flooding; b) protect or enhance water quality; c) are sympathetic to the environmental setting and the needs of the local community and d) provide a habitat for wildlife in urban watercourses (SEPA & EA, 1999). SUDS are usually made up of one or more structures to manage surface water runoff to prevent flooding and pollution. There are four general methods of control: filter strips and swales, permeable surfaces and filter drains, infiltration devices, and basins and ponds of which retention ponds will be the main focus for investigation in this study.

1.2.2 Retention ponds

Retention ponds are permanent bodies of water designed to collect surface water runoff from a large drainage catchment area (Figure 1.1). Ponds control flooding by providing flow attenuation and storage capacity of water (usually 2-3 weeks) releasing it slowly once the risk of flooding has passed (Working SUDS Party, 1999). The incorporation of emergent macrophytes such as the common reed *Phragmites australis* into retention ponds (Figure 1.2) helps to remove pollution through biofiltration and biological uptake processes (Urbonas, 1992; Working SUDS Party 1999; Revitt *et al.*, 1999). In addition, retention ponds also promote sedimentation of solids and ecological sustainability by providing a source of food for many macroinvertebrate, amphibian and bird species.

However, it must be noted that whilst biodiversity in the ponds is encouraged by engineers, they are designed to be fish free due to the negative impact fish can have on pond water quality i.e. increased faecal inputs and decreased oxygen levels.

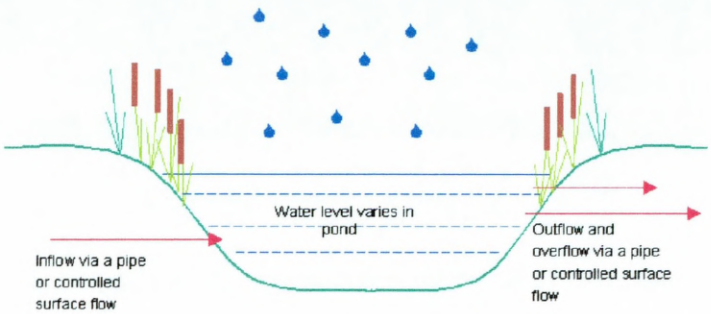


Figure 1.2 Workings of a retention pond (www.ciria.org.uk/suds.html)

1.2.2.1 The function of *Phragmites australis* in SUDS ponds

The common reed *Phragmites australis* is a tall annual grass with extensive perennial rhizomatous roots that typically penetrate to a depth of about 18 inches. Their height can range from 6 to 12 feet, with flowers of spikelets (Figure 1.3) in July to October (Campbell & Ogden, 1999). These reeds may accumulate high concentrations of potential contaminants such as heavy metals, suspended solids, nutrients and toxic organics in their roots, stems and other tissues (Whitton, 1984). This accumulation property has led to their common use in traditional wastewater treatment systems, such as sewage stabilisation ponds, to improve the water quality of municipal and industrial effluents prior to discharge. In addition, wetlands/ponds (SUDS) have been constructed with their associated flora to improve the water quality of wastewaters, urban stormwater and airfield surface runoff (Campbell & Ogden, 1999; Chong *et al.*, 1999). The wetland/pond plants improve water quality by binding soil, reducing the resuspension of muds, increasing the deposition of sediment by burial, decreasing the

water velocity and facilitating biodegradation. Additionally, biodegradation is further enhanced by algae and microbes, which are present at high densities on vegetation substrates (Lewis & Wang, 1997).

Figure 1.3 Densely growing *P. australis* close to an inlet structure from Linburn pond

Bioaccumulation by *P. australis* and associated pond flora can remove substantial quantities of toxicants and nutrients of water entering and passing through the ponds. However, the removal rates are affected by many physical and chemical factors including shading, turbidity and organic content. Generally, *P. australis* removes nutrients from the sediment during the growing season and releases them when light and temperature will not support algal growth (Lewis & Wang, 1997). Therefore, to obtain high nutrient removal rates by plant uptake, frequent harvesting of plants may be required to prevent a nutrient sink developing during the winter months (Reedy & DeBusk, 1987). In addition to bioaccumulation of toxicants and nutrients, emergent plants including *P. australis* can also provide habitats above and below water for semi-aquatic and aquatic species (Biggs *et al.*, 1995) without compromising pond function and safety as a priority.

1.2.2.2 Study Site – Dunfermline Eastern Expansion (DEX)

In the early 1990's an electronics firm entered into negotiations with Fife Council to build a manufacturing plant in the town of Dunfermline. To accommodate the industrial development area and the supporting infrastructure, the council called for a plan to convert approximately 5 km^2 of Greenfield land to a 200,000 m^2 industrial park, with supporting commercial units, a residential community of 5,500 homes together with a district parkland (Roesner *et al.*, 2001). Conventional storm drainage practises threatened to increase the frequency and volume of flooding downstream in an area with a history of flooding and create additional pollution to already stressed ecologic communities. Therefore, SUDS systems were introduced into the development to reduce the impact of urbanisation on flooding, to protect and enhance water quantity and to create wildlife habitats within an urban setting.

This site is part of a wider study undertaken by a SUDS research consortium comprising five Scottish universities, initiated in 1999, to study the applicability, acceptability and effectiveness of SUDS systems for new developments. The wider research incorporates engineering and hydrology of SUDS, and a biological/ecological assessment of SUDS retention ponds. The ponds selected for investigation were SUDS retention ponds within the DEX site. The site comprises six ponds in total, of which three were identified for study – Linburn pond, Halbeath pond and Pond 7 (Appendix 1).

1.2.2.3 Pond design structures

SUDS ponds follow certain design criteria to which SUDS engineers must adhere. Generally, retention ponds are 1.5 – 2 metres deep and contain one or more inlet, an outlet, a forebay area and under certain flow circumstances a riprap structure. Inlets are

concrete structures connected by a pipe network that drain surface water from within the catchment to a pond (Figure 1.4a & 1.6b) or another SUDS system i.e. a swale (1.2.1). Outlet pipes are located far from the inlets to allow sufficient treatment of incoming surface water and are usually positioned beneath the pond connected to a manhole outwith the pond. Treated pond water then flows from the manhole pipe to a small watercourse, i.e. a burn (Figure 1.5c). Riprap structures are made from boulders or smaller broken rock and are positioned at the face of an inlet structure to dissipate high water flows and prevent sediment resuspension at the inlet (Figure 1.4b & 1.5b). Forebay areas within ponds are located close to an inlet structure and function to trap incoming coarse sediment, decreasing the suspended solid inputs into the main treatment area. Depending on pond design, the forebay and treatment area can be separated by the common reed *Phragmites australis* (Figure 1.5a & 1.6c).

1.2.2.4 Linburn Pond

Linburn pond was constructed in spring 1998 and covers a total area of 10,200 m^2, surrounded by amenity grassland and beds of perennials, herbs and shrubs. The pond margins were planted with the common reed *Phragmites australis* and a range of low growing wetland herbs and aquatic plants have been introduced at the water's edge (Pond Action, 2000). The pond consists of four northerly inlets receiving surface water from a variety of areas within the catchment including drainage from agricultural runoff, a residential area and a building site. The outlet pipe situated in a westerly direction discharges during high flow into a small watercourse called the Lyne Burn (Figure 1.4).

a. b.

c. d.

Figure 1.4 a) view of Linburn pond; b) rip rap positioned at the front of the inlet to dissipate high water flows (Revitt *et al.*, 1999); c) inlet concrete structure showing inlet pipe with algae growing at inlet d) view of swans nesting and *Phragmites australis*.

1.2.2.5 Halbeath pond

Halbeath pond was constructed in autumn 1997 and covers a total area of 3200 m^2, surrounded by steep grassland banks. The pond margins are planted with a range of low growing aquatic wetland plants and a broad band of *Phragmites australis* (Pond Action, 2000). The pond consists of a southerly inlet receiving surface runoff from a retail development site and a westerly outlet which discharges during high flow to the Lyne Burn (Figure 1.5).

a.

b.

c.

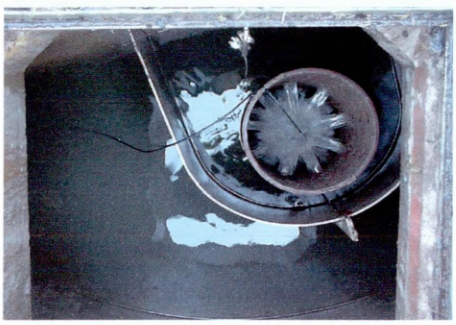


Figure 1.5 a) View of Halbeath pond showing forebay and treatment area with *P. australis*; b) Rip rap inlet with boulders to dissipate high water flows and c) Outlet pipe beneath a manhole showing outlet flow.

1.2.2.6 Pond 7

Pond 7 was constructed in autumn 1998 and covers an area of 3,100m^2. The banks are sown with grass mix and the margins planted with a range of low growing wetland herbs and aquatics with a broad band of *Phragmites australis* (Pond Action, 2000). Pond 7 was designated a control pond in this study as no housing development was planned for the catchment and surface water entering the pond was from road and agricultural runoff only. However, with the construction of a housing estate adjacent to the pond in July 2000 it is no longer a control site. The pond contains a northerly inlet receiving surface water from a housing site and a westerly inlet discharges surface water from a nearby road. The outlet structure located in an easterly direction discharges during high flow into a small watercourse called the Pinkerton Burn (Figure 1.6).

a.

b.

c.

Figure 1.6 a) view of Pond 7 from road showing *P. australis,* forebay and treatment area; b) inlet concrete structure with very low flow and c) view of Pond 7 showing housing construction on both sides of the pond.

1.3 Algae as indicators of environmental change

Algae are an ecologically important group in most aquatic ecosystems where they contribute substantially to total ecosystem primary production in most freshwater and marine habitats. Algae form the base of most aquatic food chains, produce oxygen, serve as a refuge and breeding habitat for various animals and are important in nutrient cycling (Lewis & Wang, 1997; McCormick & Cairns, 1997; Sze, 1998) (Figure 6.2). As an ecological indicator, algae meet specific indicator criteria e.g. respond rapidly and predictably to a wide range of pollutants, non-destructive sampling and therefore provide unique information concerning ecosystem condition compared with commonly used animal indicators (Dale *et al.*, 2001, McCormick & Cairns, 1994). A review by Whitton (1984) and Boyle (1984) identified certain algal genera such as *Cladophora*,

Lemanea, Enteromorpha and *Nitella* have the potential to be used as monitors of heavy metals in the aquatic environment. However, despite their potential use as early warning signals for deteriorating conditions, it was highlighted from the reviews that the possibility of routinely using algae in monitoring programmes was hampered by a lack of standardised methods for sampling, harvesting, washing and digestion. Nevertheless, in recent years, many European countries (including the Scottish Environment Protection Agency [SEPA] and Environment Agency [EA]) are using diatom-based (single-celled microscopic algae) monitoring to assess general water quality as well as acidification and eutrophication (Kelly & Whitton, 1995) in accordance with the Urban Wastewater Treatment Directive (UWWTD; 91/271/EEC) (Kelly *et al.*, 1998). The uptake of diatom monitoring throughout Europe has lead to the development of standardised "best practise" protocols (Environment Agency, 2002) allowing the straightforward transfer of data between countries. Therefore, it may be possible to adopt diatom methodology for the routine monitoring of water quality in SUDS/wetlands using algae as the indicator organism. Detailed and comprehensive studies of algae in SUDS are very limited to date. Algae are so central to aquatic ecosystem function that this thesis provides a timely insight into the potential role of algae in future SUDS development and present SUDS management.

1.3.1 Algae in waste remediation – a case study

The potential of algae as indicators of environmental change may be overlooked in many aquatic monitoring programmes. However, the usefulness of these organisms in cleaning up industrial and municipal effluents has been exploited throughout Malaysia, Thailand, USA and Europe. High Rate Algal Ponds (HRAP) have been used in the rubber industry to treat rubber effluents, where algal activities either contribute wholly

or in combination with other microorganisms to treat and purify wastewaters (Phang *et al.*, 2001). High rates of algal photosynthesis provide oxygen for aerobic bacteria which breakdown complex organic matter to simpler compounds utilised by the algae. HRAP have been successfully used in Thailand and the UK to treat sago starch wastewater and pig slurry using *Spirulina* and *Chlorella* as the algal biomass. The treated effluents were greatly reduced in chemical oxygen demand (quantity of oxygen required for the oxidation of organic compounds by strong oxidising agents), biological oxygen demand (quantity of oxygen required by bacteria for the breakdown of organic matter), ammoniacal nitrogen and phosphate levels. The resulting *Spirulina* and *Chlorella* biomass could be used as a high quality animal feed especially for aquaculture and as a source of useful biochemicals (Phang *et al.*, 2000; Fallowfield & Garrett, 1985).

Algae present in SUDS ponds may be exposed to stress (both biotic and abiotic pollutants) and in order to understand the basis and role of algae in SUDS functionality, it is important to understand algal stress responses in SUDS, not only at the applied level but also in terms of characterising the cellular and physiological basis of algal SUDS systems (Figure 1.7).

1.3.2 Effects of stress on algae

Algae, as with any other organisms, live within the constraints imposed by their environment, and when environmental conditions are extreme the algae become stressed. Stress may not only be caused by anthropogenic sources such as heavy metals, nutrients and organic compounds but can also be produced by a range of environmental factors such as changes to pH, temperature, salinity and fluctuations in light (Cox &

Norton, 1994). Stress can induce changes at the species, community and ecosystem level (Figure 1.7). In plants it has been identified that enzymes involved in xenobiotic metabolism and the antioxidant system respond sensitively to environmental pollutants. Biotransformation enzymes such as peroxidase, glutathione-s-transferase, superoxide dismutase, glutathione reductase and ascorbate peroxidase have all been suggested as markers of aquatic pollution (Seppa *et al.*, 2001). In response to possible changes that may have occurred within the ponds as a result of biotic and abiotic stress on algae, an experimental plan was devised which would measure stress indicators at the biochemical level using the green filamentous macroalga *C. glomerata* as the indicator organism (Appendix 2A & 3). This alga was chosen due to its relatively large abundance from late spring-autumn, and its dominance in all three study ponds (see Figure 1.7).

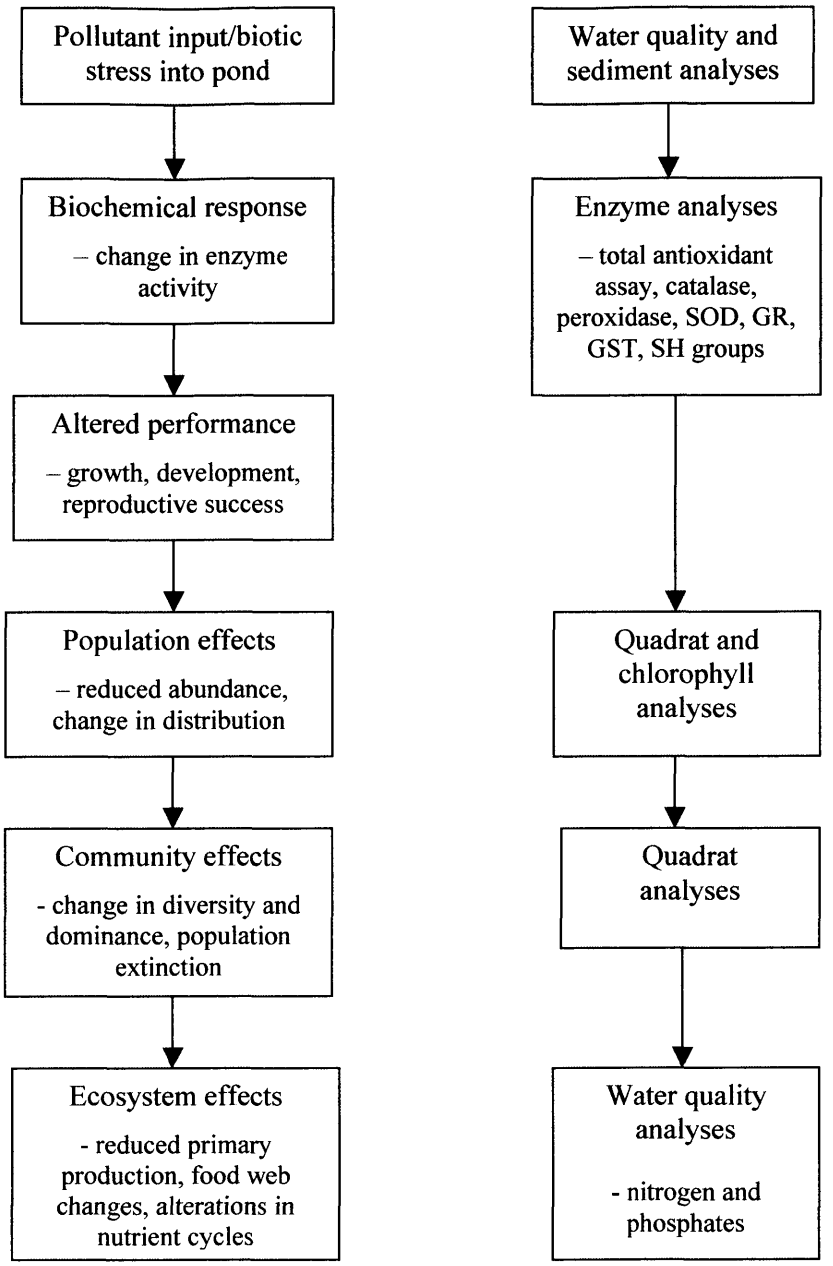


Figure 1.7 Flow diagram of effects that may be induced in algae exposed to stress (abiotic and biotic) and parameters analysed to study the effects of stress on *Cladophora glomerata* at the species, community and ecosystem level (adapted from Maltby, 1994).

SOD – Superoxide dismutase; GR – Glutathione reductase; GST – Glutathione-s-transferase; SH groups – Sulphydryl groups.

1.3.2.1 Developing criteria for assessing the biological functionality of SUDS ponds

Due to the uniqueness of this project in terms of SUDS development, it is necessary to apply individual experimental protocols that will enable algal stress responses in SUDS ponds to be ascertained. In order to fully understand the biological functionality of SUDS ponds, it is essential that the biological and chemical aspects of the aquatic pond environment are known through regular monitoring, and that this information is related to the role of algae in these systems. Therefore, experimental protocols were designed specifically for this project and include the determination of pond water quality, the distribution of algae throughout the ponds in relation to inlets and outlets and also general pond observations. Furthermore, in order to link the physiological and biochemical responses of SUDS algae to the physical pond environment, algal photosynthetic composition and antioxidant protocols were also utilised in this study. The combination of these individual approaches of study provide a novel and more "holistic" insight into the response of algae at the cellular, physiological and biochemical level in ponds.

1.3.2.2 Quadrat studies

To study the effects of biotic and abiotic stress on the individual pond algal population and algal community, a quadrat scheme was selected and has been designed to enable the abundance, diversity and dominance of algae within each of the ponds to be assessed (Figure 1.7 & Appendix 3). A quadrat is a sampling area of any shape and size; in this case the quadrat was a square of dimensions 25 cm^2. Quadrats have been used extensively in plant ecology and are one of the most common methods for counting plants (Krebs, 1985). Quadrat methodology was applied in this study to assess

the abundance and dominance of macroalgal populations within the pond perimeters. Data collected from this analysis will be related to water and sediment quality and seasonality in Chapter 2.

1.3.2.3 *Cladophora glomerata* as the algal indictor in SUDS ponds

Representatives of the genus *Cladophora* are distributed worldwide and often dominate the benthos in fresh and marine waters (Whitton, 1970). *C. glomerata* is a very common freshwater filamentous benthic chlorophyte and can usually be found as a floating mat (up to several metres long), or as a loose mass on soft substrates in many freshwater environments (Figure 1.8) (Dodds, 1991; Dodds & Gudder, 1992). Its growth may be influenced by physical and biological factors such as temperature, light, water motion and grazing (Dodds, 1991). *Cladophora* takes up nutrients from the water column where it can respond relatively rapidly to nutrient removal and nutrient availability may then be a key ecological constraint (Parker & Maberly, 2000). Previous studies have shown limitation by nitrogen (Lohman & Priscu, 1992) and most commonly phosphorous (Planas *et al.*, 1996). Factors normally associated with the dominance of *Cladophora* in aquatic ecosystems are: high light, pH > 7.0, high calcium and high dissolved P:N ratios (Dodds, 1991). Furthermore, the tough and flexible thallus (body of an alga) of *Cladophora* makes this species ideally suited to rivers and habitats with high water flow (Dodds, and Gudder, 1992). Dense growth can clog water works, resulting in excessive diurnal swings in O_2 levels and are not aesthetically pleasing. However, it has been reported that *Cladophora* is one of the most sensitive algae to heavy metals pollution where its presence in aquatic environments indicates that this pollution is not having a marked influence on the ecosystem (Whitton, 1979).

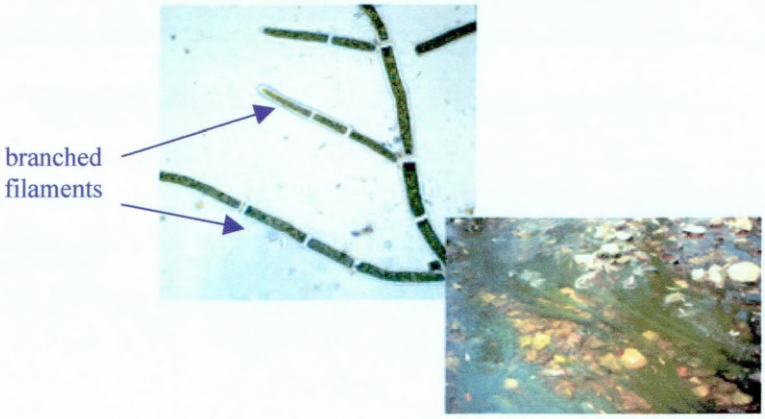


Figure 1.8 Magnified (x100) *C. glomerata* filament and attached to a substrate in the aquatic environment (Vis *et al.*, 1994).

1.3.2.4 Control of algae using barley straw and Duckweed

Dense growths of filamentous algae can pose a number of problems in water such as impeding flow in drainage systems, blocking pumps, interfering with fishing and other forms of amenity and, in some instances, creating a health hazard to humans, livestock and wildlife as some algae are toxic (Codd *et al.*, 1989; Codd, 2000). Due to their small size and rapid growth rates, algae are difficult to control by methods used for other aquatic plants. Mechanical methods (raking, cutting, harvesting) are largely unsuccessful due to the large numbers of fragments that remain and rapid algal regrowth. Many algae are susceptible to herbicides but these prove unpopular as they kill many submerged aquatic plants (Caffrey & Monahan, 1995, 1999). A relatively new method of algal control involves the application of barley straw to water body (Martin & Ridge, 1999). This method offers a cheap and environmentally acceptable method of algal control. When straw rots, the cell walls decompose to release lignin, which is then oxidised to humic acid and other humic substances under aerobic conditions. Humic substances react with dissolved oxygen in water in the presence of sunlight, to produce hydrogen peroxide. Peroxides are short lived, but a continual supply of humic substances from slowly decomposing straw results in peroxides being

continuously generated whenever there is sufficient sunlight (Pillinger *et al.*, 1994; Ridge & Pillinger 1996; Barrett *et al.*, 1996). It is this action that causes algal growth to be inhibited. However, sensitivity of algae to decomposing straw varies between algae, and is not related to general taxonomic or structural characteristics (Martin & Ridge, 1999). Diatoms appear to be less resistant to the inhibitor (Ridge *et al.*, 1995); however, planktonic diatoms are suppressed after the introduction of barley straw (Martin & Ridge 1999). Growth of the filamentous macroalga *C. glomerata* has been demonstrated to be susceptible to barley straw treatment (Caffrey & Monahan, 1999; Martin & Ridge, 1999; Ridge & Pillinger, 1996) where the treatment does not appear to have any effect on higher plants, invertebrates or fish (IACR information sheet 3, 1999).

An additional method of algal control is to introduce the small aquatic plant Duckweed into a water body. Duckweed (*Lemna, Spirodela, Wolffia spp.*) are floating aquatic plants that grow very fast and cover the water surface (Figure 1.9) (Hancock & Buddhavarapu, 1993; Campbell & Ogden, 1999). A surface layer of these plants can form a physical barrier and thus prevent light penetration into the water column except for the top few centimetres. With the elimination of light, algae die due to a lack of light for photosynthesis. However, growth of these plants must be controlled, as reduced light penetration into the water column can inhibit growth or kill many submerged aquatic plants.

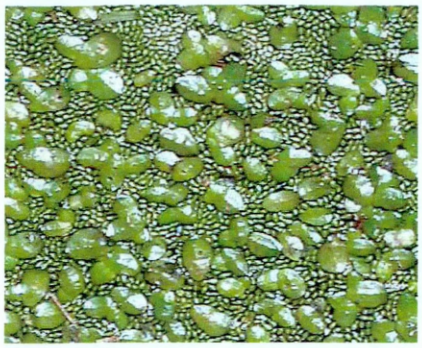


Figure 1.9 Photograph of floating Duckweed mat covering the pond water surface

Importantly, a balance must be reached between the positive and negative effects of algae in aquatic systems. Literature focuses too often on the 'bloom problem' in aquatic ecosystems rather than the positive roles algae have too offer such as the possibility of utilising algae in phytoremediation programmes (1.3.1) and in primary production.

1.3.2.5 Algal photosynthetic pigments

For photosynthesis to occur in algae, pigments absorb light that is converted into chemical energy and then used to synthesise organic compounds from carbon dioxide (Sze, 1998). "Usable light" for photosynthesis is in the 400 – 700λnm electromagnetic spectrum where individual pigments selectively absorb certain wavelengths of photosynthetically active radiation (PAR). The primary photosynthetic pigment in all oxygen evolving photosynthetic organisms is chlorophyll *a*. Chlorophyll *a* shows two main absorption bands *in vitro* where one band is in the red light region near to 660 nm and 665 nm and the other is in the Soret region near to 430 nm (Meeks, 1974). In addition to chlorophyll *a*, other algal chlorophylls are present in algae but they have limited distribution and are generally considered accessory pigments. Chlorophyll *b* is found in the Chlorophyceae algal division (includes *C. glomerata*) (Allen 1966; Kirk 1983; Sze, 1998 and Van den Hoek *et al.*, 1995) and functions as a light harvesting

pigment, transferring absorbed light energy to Chlorophyll *a* for primary photochemistry. Chlorophyll *b in vitro* shows two main absorption maxima with one in the red region near to 645 nm and the other in the Soret region near to 435 nm.

It is known that the chlorophyll content of algae can be influenced by light intensity and research carried out by Kirk & Tilney-Bassett (1967) showed that within certain limits, the chlorophyll content of algae is inversely proportional to light intensity during growth i.e. as light intensity during growth decreases, the content of the photosynthetic pigments can increase and vice-versa (normally two- to five-fold increases are observed) (Kirk, 1983; Meeks, 1974). It has been hypothesised that the regulation of chlorophyll is due to an accumulation of a photosynthetic product under non-limiting light conditions that may inhibit chlorophyll synthesis (Beale & Appleman, 1971). However, studies of chlorophyll *a/b* ratios and light intensity by Reger and Krauss (1970) on the alga *Chlorella vanniella* identified that an increase in light intensity can result in a rise in that ratio. Therefore, deviations from the normal chlorophyll *a/b* ratio of chloroplasts for *C. glomerata* of 2.6:1 (Larkum & Barrett, 1983) can indicate exposure to high/low light intensity and fluctuating light penetration levels related to turbidity in the ponds.

In addition to chlorophyll, algae contain other photosynthetic pigments, such as carotenoids, which may participate in photosynthesis but additionally function in photoprotection (Sze, 1998). Exposure to high levels of light for prolonged periods may inhibit photosynthesis and even damage the photosynthetic system (Dodds & Gudder, 1992). To reduce such adverse affects, some carotenoids function in photoprotection by screening the chloroplasts or by absorbing and dissipating excess energy (Sze, 1998).

1.4 Water chemistry

In order to gain an understanding of the level of primary production and alterations in the nutrient status of the ponds throughout the study period, it was necessary to study pond water quality on a frequent basis. This experimental approach will help to determine pond pollutant loads and nutrient status, which in turn will subsequently assist in the understanding of the response of algae to stress at the cellular, physiological and biochemical level in ponds (Figure 1.7). The water quality parameters selected for this study follow the general sanitation analyses routinely undertaken by water authorities and monitoring water bodies.

1.4.1 pH

pH is a measure of the concentration of hydrogen ions (H^+) in an aqueous solution. It is an important variable in water quality assessment as it can influence many biological and chemical processes within a water body. The pH scale runs from 0 to 14 (i.e. very acidic to very alkaline) with pH 7 representing a neutral condition. In unpolluted waters, pH is principally controlled by the balance amongst carbon dioxide, carbonate and bicarbonate ions as well as other natural compounds such as humic and fulvic acid (Gray, 1999). Daily variations in pH can be caused by the photosynthesis and respiration cycles of algae especially in eutrophic waters (Chapman, 1996). Generally, the pH of natural waters is between 6.0 to 8.5 where most freshwater algae grow best in neutral to slightly alkaline conditions (Kozitskaya & Komarenko, 1995) including the macroalga *C. glomerata* which favours growth in pH > 7.0 (Pitcairn & Hawkes, 1973; Dodds, 1991).

1.4.2 Conductivity and chloride

Analysing for conductivity and chlorides in surface waters can give an indication of the level of pollution from industrial and sewage effluents and also from both agricultural and road runoff entering the ponds (Chapman, 1996). Conductivity is a measure of the ability of water to conduct an electric current and because it is linked to the concentration of mineral salt (Cl^-, SO_4^{2-}, K^+ and Na^+) in solution, it can be related to chloride concentration (Chapman, 1996; Gray 1999). The action of road salting during the winter and early spring months can contribute significantly to massive increases in both chloride and conductivity concentrations in surface waters. During these months, conductivity and chloride concentrations can exceed > 1,000 $\mu S.cm^{-1}$ and > 2 $mg.l^{-1}$ respectively (Chapman, 1996; Gray, 1999). These substantial fluctuations may have an affect on the overall health of the aquatic ecosystem and consequently algae may respond to the variation at the biochemical, cellular or physiological level.

1.4.3 Suspended solids

It is important to analyse suspended solids (SS) in surface waters as they can have a major impact on the primary production of freshwaters. This is because of their light attenuating properties and they can therefore affect phytoplankton, benthic algae and macrophytes (Maitland, 1990; Gray 1999; Novotny, 1998). Suspended solids may be classed as insoluble or soluble particles, too large to dissolve quickly and too small to settle out of suspension under the prevailing turbulence and temperature (Gray, 1999). Suspended solids consist of silt, clay, fine particles of organic and inorganic matter and soluble organic compounds (Chapman, 1996). These particles can vary in size from approximately 10 nm in diameter to 0.1 mm in diameter. The type and concentration of suspended solids can control the turbidity and transparency of water. Light penetration

in water can be severely reduced such that photosynthesis is only possible in the upper layers and silts, which settle on the bottom, may blanket out algae and rooted macrophytes (Maitland, 1990). Suspended solids concentrations < 50 $mg.l^{-1}$ are unlikely to cause adverse effects on fish health (Alabaster, 1980). However, very high levels of suspended solids are usually episodic in nature and are related to high rainfall and floods in the catchment. Generally, the water clears after these episodes where most of the material settles on the bottom, is gradually washed away, or is incorporated into the normal substrate (Maitland, 1990).

1.4.4 Nitrogen compounds

Nitrogen is an essential compound for all living organisms as it is an important constituent of proteins and many essential macromolecules. In the environment, inorganic nitrogen occurs in a wide range of oxidation states such as nitrate (NO_3^-) and nitrite (NO_2^-), the ammonium ion (NH_4^+) and molecular nitrogen (N_2). When molecular nitrogen is dissolved in aqueous systems, it is converted to aqueous forms of inorganic nitrogen such as nitrate, the most common form of nitrogen (NO_3^-), followed by ammonia (NH_3) and then nitrite (NO_2^-). These forms of nitrogen, with the exception of NO_2^-, are readily available for biological activity and are actively taken up by plants and microorganisms, which then convert inorganic nitrogen, back to organic nitrogen (Waite, 1984).

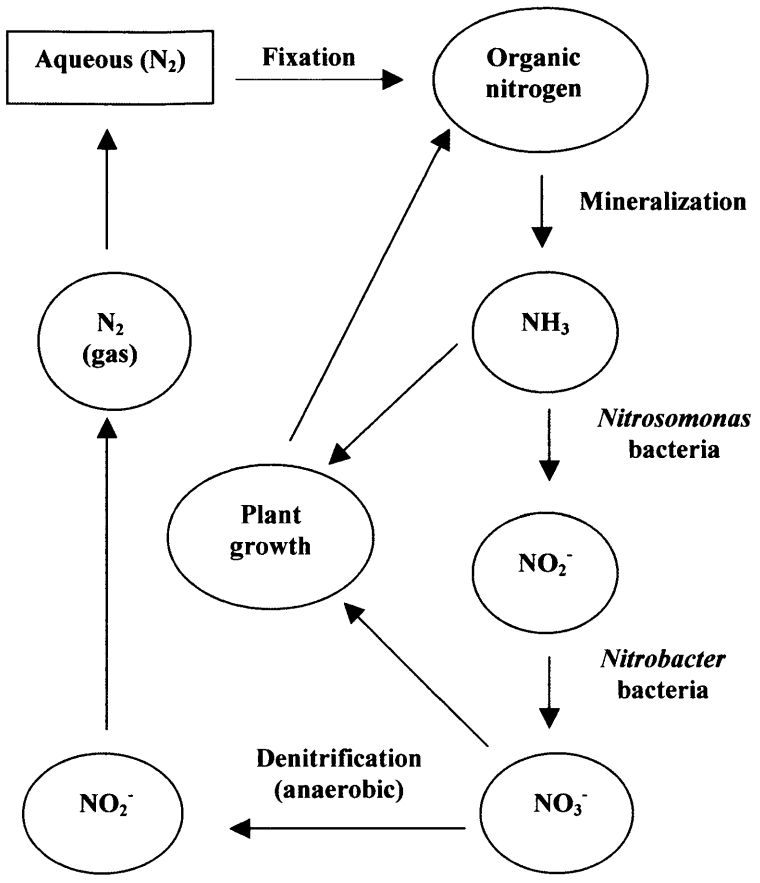


Figure 1.10 The nitrogen cycle (adapted from Waite, 1984)

1.4.4.1 Ammoniacal nitrogen (NH_3-N)

Ammonia occurs naturally in a water body and arises from the breakdown of nitrogenous organic and inorganic matter in soil and water, excretion by biota, reduction of nitrogen gas in water by microorganisms and from gas exchange with the atmosphere (Chapman, 1996). Nitrification of ammonia can consume large quantities of oxygen. Therefore high concentrations of ammoniacal nitrogen can be toxic to aquatic life and detrimental to the ecological balance of water body (Davis *et al.*, 1991; Sawyer *et al.*, 1994). Unpolluted waters usually contain < 0.1 mgl^{-1} ammoniacal nitrogen and ammonia compounds. However, surface waters can reach 2-3 $mg.l^{-1}$, which at these concentrations, generally indicates organic pollution from domestic sewage, industrial waste and fertiliser runoff (Chapman, 1996).

1.4.4.2 Nitrite and nitrate

The nitrate ion (NO_3^-), originating from the breakdown of nitrite (NO_2^-) by *Nitrobacter* bacteria under aerobic conditions, is the most common form of combined nitrogen in natural waters (Chapman, 1996). Nitrate is an essential nutrient for aquatic plants and algae where seasonal fluctuations can be caused by plant/algal growth and decay. Natural nitrate concentrations can be enhanced by the use of inorganic nitrate fertilisers and from municipal and industrial wastes. Concentrations of nitrate in excess of 0.2 $mg.l^{-1}$ tend to stimulate algal growth and indicate possible eutrophic conditions. However nitrate concentrations in freshwaters rarely exceed 1 $mg.l^{-1}$ (Sawyer *et al.*, 1994; Chapman, 1996). When influenced by human activities, surface waters can have nitrate concentrations > 5 $mg.l^{-1}$ which usually indicate pollution from human/animal waste or fertiliser runoff and are often associated with unsatisfactory microbiological quality of water. Determination of nitrate plus nitrite (Total Oxidised Nitrogen) can give a general indication of the nutrient status and level of organic pollution in aquatic systems.

1.4.5 Phosphorous compounds

Phosphorous is an essential nutrient for living organisms. However, it is also the limiting nutrient for algal growth, and so controls the primary productivity of a water body (Davis *et al.*, 1991). In natural waters, phosphorous occurs mostly as dissolved orthophosphates, polyphosphates and organically bound phosphates (Chapman, 1996). Natural sources of phosphorous are mainly associated with the weathering of phosphorous-bearing rocks and the decomposition of organic matter. Domestic waters (containing detergents), industrial effluents and fertiliser runoff also contribute to elevated levels in surface waters. Phosphorus is taken up by algae/plants in the

inorganic form (PO_4^{3-}) and is incorporated into organic compounds. During algal/plant decomposition, phosphorus is returned in organic form and usually deposits in the sediments where it is rehydrolized by bacteria and released back into the water column in its available inorganic form (Chapman, 1996; Davis *et al.*, 1991). Phosphorous is rarely found in high concentrations in freshwaters as plants and algae actively take it up and as a result there can be considerable seasonal fluctuations in phosphate concentrations in surface waters (Chapman, 1996). The increase in phosphorus levels in water from anthropogenic sources has been considered the single most important factor changing the balance of productivity in freshwater and is largely responsible for eutrophic conditions (Round, 1981; Chapman, 1996).

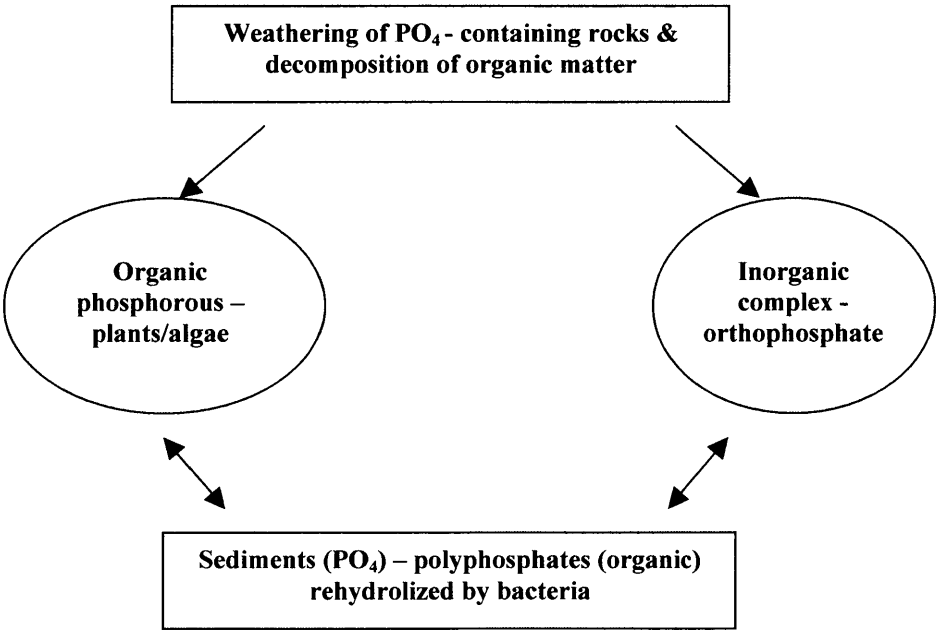


Figure 1.11 The phosphorous cycle (adapted from Waite, 1984)

1.4.5.1 Eutrophication & primary productivity

Eutrophication is the process by which bodies of water become more enriched and productive and is initiated by the addition of macronutrients such as nitrogen and phosphorous to water systems (Waite, 1984). Inputs of these nutrients may come from domestic, industrial, agricultural waste and also from stormwater runoff (Waite, 1984). Nitrogen and phosphorus washed from lawns and streets during storms may be transported directly to water systems. The main effects of eutrophication in a water body are a) blanketing of the sub-strata resulting in a reduction in habitat diversity; b) reduction of oxygen concentration and light penetration in water; c) formation of algal blooms resulting in harmful algal toxins, water discolouration, offensive taste and odour and d) increased macrophytic and filamentous algal growth which impede water flow, reducing the recreational value of a water body and increasing oxygen demand as a result of plant/algal decay (Gray, 1999; Lewis & Wang, 1997; Waite 1984).

The nutrient status of a water body can play an important role in determining the primary productivity of an aquatic system. Primary productivity refers to the ability of a water body to sustain a food web and is linked to the amount of algal growth that can be supported by the available nutrients (Davis *et al.*, 1991). Increased productivity can reduce water quality due to enhanced algal growth. Therefore, the water quality of an aquatic system can be classified according to its productivity into one of three trophic categories - oligotrophic, mesotrophic and eutrophic. Oligotrophic conditions indicate non-productive water and are associated with low biological activity; mesotrophic conditions indicate water with average productivity and are associated with some biological activity but are still a balanced system and eutrophic conditions indicate

highly productive water with excessive biological activity causing large fluctuations in environmental parameters (Waite, 1984).

In order to link the physiological and biochemical response of the bioindicator alga to abiotic and biotic stress in urban ponds, and relate these responses to help identify pond health, this study will incorporate a novel experimental approach. This uses a generic stress response based on free radical mediated oxidative stress, to determine the responses of a "test" alga, *C. glomerata* to environmental impacts.

1.5 Free radicals and reactive oxygen species (ROS)

A free radical can be defined as any species that contains one or more unpaired electrons (Benson, 1990). The presence of an unpaired electron means that free radicals are (a) highly reactive and (b) paramagnetic (containing magnetic properties). Free radicals can be formed by the addition of an electron to a neutral molecule, the loss of an electron from a neutral molecule or cleavage of a bond (two electrons) between atoms so that each species receives an electron (Benson & Bremner, 2003).

Oxygen, the primary agent for aerobic respiration and metabolism has the potential to be an important promoter of cellular damage in aerobic organisms (Benson, 1990). Although molecular oxygen is completely harmless, its toxicity comes from the products of its reduction and consequently this action promotes the production of cascading damaging chain reactions (Benson & Withers, 1987; Benson & Roubelakis-Angelakis, 1994). Molecular oxygen is a biradical, possessing two unpaired electrons of parallel spin. This configuration is termed ground state or triplet state ($^3O_2$) and it describes the electronic and paramagnetic behaviour of oxygen. If energy, usually in

the form of ultraviolet light, is imparted to normal triplet state oxygen it is converted into a much more reactive (although not a radical) singlet oxygen ($^1O_2$) (Figure 1.12).

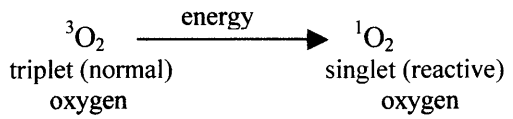


Figure 1.12 Formation of singlet reactive oxygen (Benson & Bremner, 2003)

To produce two molecules of H_2O, molecular oxygen must accept four electrons, but because of spin restrictions, O_2 cannot accept four electrons at once and therefore accepts one electron at a time. Thus, during the stepwise reduction of O_2, intermediates are formed such as the reactive oxygen species superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) (Figure 1.13).

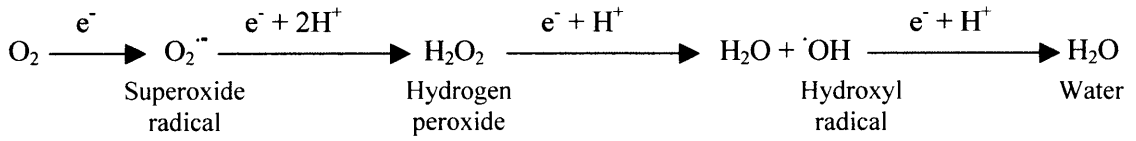


Figure 1.13 Stepwise formation of reactive oxygen species (Mallick & Mohn, 2000)

In plant systems, ROS are always formed by the inevitable leakage of electrons onto molecular oxygen from the electron transport activities of chloroplasts, mitochondria and plasma membrane (Fridovich, 1995; Foyer, 1997). In addition to normal metabolic processes, ROS in higher plants and algae are also stimulated by various environmental stresses such as exposure to levels of high light (Foyer *et al.*, 1997, drought (Smirnoff, 1993), heavy metals (Weckx & Clijsters, 1996), high salt concentration (Meneguzzo *et al.*, 1999) and extremes of temperature (Doke *et al.*, 1994; Fleck, 1998). In algae, ROS can adversely affect vital functions such as photosynthesis and respiration leading to

membrane damage and loss of integrity (Scandalios, 1997). Therefore, the involvement of ROS may be of relevance to *C. glomerata* collected from urban ponds exposed to various environmental stresses.

There is a close relationship between ROS and transition metal ions (Fe^{2+}/Fe^{3+}), which are involved in both radical generation and scavenging, i.e. the dismutation of superoxide. In the presence of Fe^{2+} catalyst, the Haber-Weiss and Fenton reactions play an important role in producing the highly toxic hydroxyl radical ($^{\cdot}OH$) (Benson, 1990). Haber proposed a reaction between superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2).

$$H^+ + H_2O_2 + O_2^{\cdot-} \longrightarrow O_2 + H_2O + {}^{\cdot}OH$$

However, the original reaction was later found to occur in two steps known as the Fenton reaction. Firstly, the superoxide radical reduces the ferric ion:

$$Fe^{3+} + O_2^{\cdot-} \longrightarrow Fe^{2+} + O_2$$

Secondly, this interacts with H_2O_2 to produce the $^{\cdot}OH$ radical:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$$

The sequence of events for the formation of highly reactive hydroxyl radical ($^{\cdot}OH$) can be summarised as follows:

$$O_2^{\cdot-} + H_2O_2 \xrightarrow[\text{catalyst}]{Fe^{3+}/Fe^{2+}} O_2 + {}^{\cdot}OH + OH^-$$

Biotic and environmental stresses lead to increases in cellular oxidants that induce the increased synthesis of highly specialised metabolites (antioxidants) to help protect against oxidative damage (Foyer *et al.*, 1997). Induction of antioxidant defences can be

assumed to reflect a general strategy required to overcome increased oxidative stress due to the pressure of environmental constraints. Antioxidants confer protection by quenching or scavenging the ROS and are usually dependent upon the scavenger being in close proximity to the site of ROS production (Benson, 1990). Antioxidant defence to oxidative damage is achieved by both enzymatic and non-enzymatic antioxidants.

Algae contain an extensive range of antioxidants both enzymatic and non-enzymatic which help to reduce or remove potentially damaging free radicals. It is this extensive antioxidant capacity, which enables algae to inhabit, survive and remediate polluted aquatic environments.

1.5.1 Non-enzymatic antioxidants

The carotenoid pigments in photosynthetic organisms form the first line of defence against free radical damage in chloroplasts (Benson, 1990, Mallick & Mohn, 2000). Carotenoids protect the photosynthetic apparatus from excess light by allowing excited chlorophyll molecules to pass on their energy to carotenoid pigments in preference to O_2 therefore preventing the formation of singlet oxygen ($^1O_2$). In addition, carotenoids are also able to quench singlet oxygen ($^1O_2$) and provide a significant protective pathway in non-photosynthetic membrane systems (Benson, 1990 & Mallick & Mohn, 2000).

^{3}chlorophyll* + O_2 ⟶ chlorophyll + $^1O_2$
(excited triplet state) (singlet oxygen)

^{3}chlorophyll* + ^{1}carotenoid ⟶ ^{1}chlorophyll + ^{3}carotenoid
(singlet state) (decays within msecs)

$^1O_2$ + ^{1}carotenoid ⟶ $^3O_2$ + ^{3}carotenoid
(triplet normal oxygen)

Vitamin E (α-tocopherol) when embedded into membranes is in close proximity to potentially vulnerable sites of free radical damage. α-tocopherol is therefore preferentially oxidised during ROS attack as compared to polyunsaturated fatty acid (PUFAS) (Benson & Bremner, 2003). In addition, vitamin E has also been found to deactivate singlet oxygen $^1O_2$ (Takenaka *et al.*, 1991). Vitamin C plays an important role in reducing superoxide and hydroxyl radicals and also in the regeneration of vitamin E (Alscher *et al.*, 1997; Mallick & Mohn, 2000). Regeneration of vitamin E is thought to occur via an ascorbate cycling mechanism, where α-tocopherol quinone is reduced by ascorbic acid (vitamin C) back to α-tocopherol (Packer *et al.*, 1979; Kunert & Ederer, 1985).

1.5.2 Enzymatic antioxidants

Superoxide dismutase (SOD) comprises a family of metalloisoenzymes that contain manganese, iron or copper and zinc as their prosthetic metals (Benson, 1990; Salin, 1987). SOD is an antioxidant enzyme widely distributed among prokaryotes and eukaryotes. In plant tissues, most of the SOD can be detected in the chloroplasts due to their susceptibility to oxygen toxicity and in non-photosynthetic tissues it is found in the cytoplasm and mitochondrial membranes (Halliwell, 1982). SOD antioxidant enzymes catalyse the reaction of the superoxide radical ($O_2^{\cdot-}$) to oxygen (O_2) and hydrogen peroxide (H_2O_2). In plants, H_2O_2 is produced not only by the SOD reaction but also during photorespiration (Benson, 1990). In other cellular compartments, H_2O_2 degradation is largely performed by the action of catalase and glutathione peroxidase. Glutathione peroxidase reduces not only H_2O_2 but also organic peroxides (products of ROS reactions with organic molecules) (Bartosz, 1997). Catalase is a haeme-containing enzyme found in all aerobic organisms and catalyses the breakdown of H_2O_2 and the

oxidation of hydrogen donors (Packer, 1984). However, not all the H_2O_2 produced in plant cells is dissipated by the action of catalase. Several different peroxidases also catalyse the dissipation of H_2O_2. Ascorbate peroxidase plays an essential role in scavenging hydrogen peroxide in chloroplasts (Asada & Takahashi, 1987) and thus protects cell membranes and membrane-bound enzymes from damage and loss of cell integrity (Ishikawa *et al.*, 1993). In addition, guaiacol peroxidases found in plants, participate in many physiological processes such as the biosynthesis of lignin and auxin degradation (Salin, 1987; Asada, 1992).

Glutathione (GSH), a low molecular weight tripeptide found in the vast majority of prokaryotic and eukaryotic cells, and is usually the most abundant intracellular non-protein reduced sulphur thiol (S-H) (Noctor *et al.*, 1998; Anderson, 1985). GSH plays an important role in the defence of plants and other organisms against oxidative stress, participates in ascorbate recycling and acts as a substrate for the multifunctional antioxidant enzyme glutathione-s-transferase (Noctor *et al.*, 1998). GSH prevents enzyme inactivation by protecting potentially susceptible protein thiol groups from oxidative degradation (Foyer & Halliwell, 1976; Halliwell & Foyer, 1978; Halliwell, 1982; Packer, 1984; Alscher, 1989). Protection by GSH is achieved by providing a preferential substrate for S-H oxidation. The product of this reaction is oxidised glutathione (GSSH), which if permitted to accumulate can prove toxic to the cells. However, accumulation is controlled by glutathione reductase which catalyses the degradation (reduction) of GSSG back to the protective GSH molecule (Benson, 1990). In addition, GSH protects the chloroplasts from oxidative damage by removing H_2O_2 in conjunction with ascorbate recycling (Halliwell, 1982). Furthermore, glutathione-s-transferase (GST) catalyses the conjugation of GSH with potentially damaging

xenobiotics (Marrs, 1996) rendering them non-toxic and can also reduce damaging lipid peroxides to non-toxic hydroxides (Habig *et al.*, 1974).

1.5.3 Sulphydryl groups

There are two types of sulphydryl groups in proteins the non-protein thiols represented by very low molecular weight compounds such as amino acids (cysteine) or glutathione (GSH) and the high molecular weight protein thiols (protein bound sulphydryls) (Faure & Lafond, 1995). Sulphydryls play several important roles in proteins including the conformation of structure, acting as the catalyst at the reactive site of numerous enzymes (e.g. thiol protease), binding for the substrate of some enzymes, binding of some subunits, conformational change linked with allosteric processes and in a protective role against free radical effects (Faure & Lafond, 1995). The non-protein sulphydryl compound glutathione (GSH) acts as an important redox buffer, conferring protection to oxygen-sensitive enzymes and susceptible protein thiol groups from oxidative damage by providing a preferential substrate for S-H oxidation (Halliwell, 1982; Packer 1984; Alscher, 1989). In addition, glutathione also protects cells against ionizing radiation (Held & Hopcia, 1993). Numerous authors have speculated that protein thiols could be an intermediate reservoir protecting against free radical attack due to their ability to react with hydroxyl radicals (·OH) near to SH sites and to scavenge superoxide radicals ($O_2^{\cdot -}$) (Faure & Lafond, 1995). Furthermore, the oxidation of protein thiols also protects cells against the damaging effects of nitric oxide (NO_2) and ozone (O_3) (Halliwell *et al.*, 1992; Chevrier *et al.*, 1988).

1.5.4 Analytical techniques used to measure ROS activity

The development of analytical techniques that measure ROS activity in algae will provide a greater insight into the oxidative stress response of algae to biotic and abiotic pressures in the urban pond environment. These techniques also permit *in vitro* assays to be performed on selected algal species using specific contaminants identified in the urban pond and can thus mimic the biochemical response generated *in vivo*.

The complexity and rapidity of free radical reactions can pose analytical problems and so it is important to monitor oxidative stress as well as changes in antioxidant status. Assays monitoring the activity of free radical reactions and associated compounds have given a much-needed insight into the activity of free radicals within active tissues. Several assays have been developed and employed to profile the extent of oxygen-derived free radicals within biological samples and the secondary products produced when they react with cellular components. A number of these assays utilise spectrophotometry techniques to measure the activity of free radicals such as SOD (Beuchamp & Fridovich, 1971) and some measure antioxidant activity such as catalase and peroxidase (Aebi, 1983; Castillo *et al.*, 1984). The development of a total antioxidant assay that can measure changes in the total antioxidant status of biological samples exposed to oxidative stress would be beneficial for large-scale monitoring programmes as it can identify samples of interest quickly. Such an assay has been developed for macroalgae (3.2.2.5 & 3.2.4) and is based on a simple spectrophotometry decolourisation assay whereby a 2,2' –azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) radical is generated directly and reacted with potassium persulfate to produce a blue/green chromophore. The $ABTS^{\cdot+}$ chromophore is then reduced to ABTS (non-radical) by antioxidants present in biological samples and can be measured as a

decrease in absorbance at 734nm. The extent of reduction is dependent on antioxidant activity, concentration of the antioxidant and duration of the time series reaction (Re *et al.*, 1999).

$$\text{ABTS} + \text{potassium persulfate} \longrightarrow \text{ABTS}^{\cdot+} + \text{antioxidants} \xrightarrow{734\text{nm}} \text{ABTS}$$

Furthermore, indirect monitoring of free radicals using ROS 'markers' can also provide further evidence of free radical damage in biological samples. The indirect and non-invasive monitoring of ROS 'markers' was successfully used for the microalgae *E. gracilis* and *H. pluvialis* exposed to ultra low temperatures (Fleck, 1998; Fleck *et al.*, 2000) and monitors the level of hydrocarbons emitted when biological tissues are exposed to stress in the presence of dimethyl sulphoxide (DMSO) (Benson & Withers, 1987). The technique is based on the generation of hydroxyl radicals ($^{\cdot}OH$) formed as a result of oxidative stress reacting with DMSO (hydroxyl radical scavenger) to produce methane, which can be detected by volatile headspace sampling and gas chromatography (Harding & Benson, 1995; Fleck 2000).

1.5.5 Study rationale

This thesis comprises several integrated and parallel studies of pond attributes (see Figure 1.7) and biochemical investigations. The thesis is structured as separate investigative chapters of which the interpretation of findings (Chapters 2 – 5) is presented on an individual chapter basis. The final discussion (Chapter 6) aims to not only summarise the overall findings of the study but to integrate the outcomes of each investigative approach. The final component of the study (Chapter 6) therefore concludes with a series of management and future recommendations.

Chapter 2 AN ENVIRONMENTAL ASSESSMENT OF THE PHYSICAL, CHEMICAL AND BIOLOGICAL INTERACTIONS IN SUDS PONDS

2 Introduction

The objective of this project was to explore the use of algae as an indicator organism of biotic and abiotic change in urban aquatic environments. Interpretations will be aimed at defining and managing the biotic component of SUDS ponds. This chapter focuses on the development of qualitative and quantitative techniques, which were used to analyse and elucidate biological interactions within SUDS ponds. The chapter will comprise specific pond assessments including the analysis and determination of pond water quality, algal chlorophyll composition and distribution within the ponds, general pond status and health observations and site visits. The pond sites selected for study are used for the management of agricultural, industrial and urban runoff. The determination of pond water quality will provide an indication of pollutant loads and consequently the xenobiotic stress to which the algae are exposed. In addition, algal chlorophyll compositions, which are an important biochemical marker in algae, will be analysed therefore providing information on algal chlorophyll content/ratios that may be related to stress impacts. Furthermore, the distribution of the common macroalgae *C. glomerata* throughout the pond will be assessed to map particular 'hot spot' areas of algal accumulation and related to previously collected sediment data generated by the SUDS project. Data from this chapter supports the rest of the thesis and by combining the environmental assessments of SUDS ponds, will result in a greater understanding of the biological and chemical interactions within urban ponds. This knowledge can lead to the development of improved SUDS pond management strategies for biological

sustainability and the enhancement of plant and animal varieties (biological biodiversity) (Lloyd, 2002) within the urban landscape.

2.1 Traditional urban drainage versus SUDS systems

Traditional methods of urban drainage i.e. a Combined Sewerage System (sewage plus surface water) were viewed by local authorities as an unsustainable practise due to their cost, energy use, pollution of watercourses, degradation of waterways and damage caused to the urban landscape. Therefore, local authorities introduced a new and improved method of urban drainage in the mid 1990's to provide a cheaper alternative to the ageing and insufficiently combined sewerage systems. The new practise eliminated surface water from sewerage systems and treated the surface water at source. The introduction of these structures, termed Sustainable Urban Drainage Systems (SUDS) were considered a more sustainable option to traditional methods of control due to their reduced cost, energy use, improved water quality through biological treatment, reduced flooding and land intake. Moreover, SUDS, if managed and designed effectively, reduce ecological damage to the urban landscape and create wildlife habitats within urban areas, thereby enhancing biological biodiversity (Working SUDS Party, 1999; Everard & Street, 2001).

SUDS comprise structures that are built to manage surface water runoff, providing storage or attenuation of water and exploiting the natural processes of sedimentation, filtration and biodegradation to remove pollutants, whilst creating and improving wildlife habitats within urban areas (http://www.ciria.org.uk/suds.html).

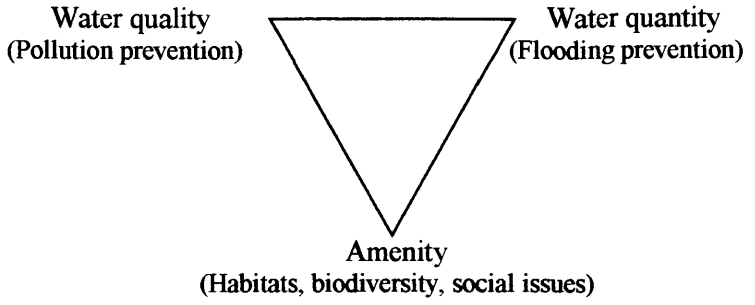


Figure 2.1 The Urban Drainage Triangle (Working SUDS Party, 1999)

The natural uptake of selected pollutants by plants and algae (phytoremediation) is potentially one of the most important biological properties of SUDS ponds, as this treatment process can be utilised not only for urban runoff, but for other polluted discharges such as sewage, using a similar treatment pond system such as reed beds. Therefore, SUDS implementation into the urban landscape may pave the way for future technological developments of 'ecologically friendly and more sustainable' treatment systems for potentially hazardous discharges.

2.1.1 Retention ponds

Retention ponds are structures designed to collect surface water runoff from a large drainage catchment area. Ponds control flooding by providing flow attenuation and storage capacity of water (usually 2-3 weeks) releasing it slowly once the risk of flooding has passed (Working SUDS Party, 1999). Ponds also treat runoff by providing settlement of solids in the sediments, adsorption by aquatic vegetation or the soil and biodegradation to remove pollutants. Furthermore, ponds also offer ecological sustainability since they can maintain aquatic food webs and provide a source of food for many macroinvertebrates, amphibian and bird species.

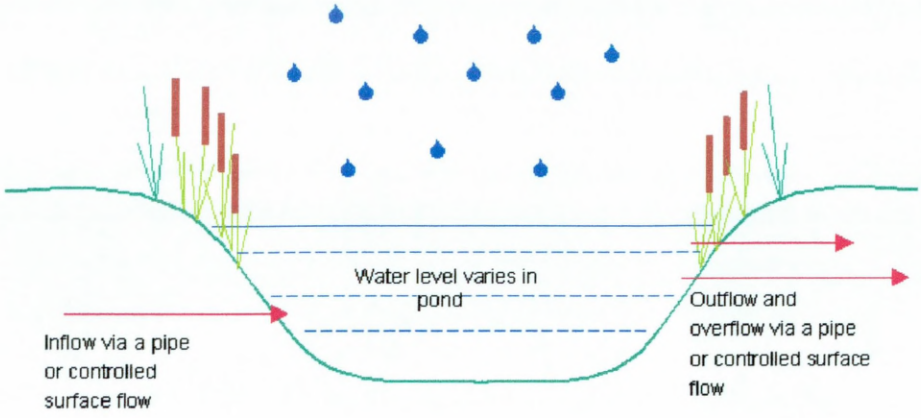


Figure 2.2 Workings of a retention pond (http://www.ciria.org.uk/suds.html)

2.1.2 Pond study sites

The study ponds for this project are SUDS retention ponds which function to control flooding, improve water quality and are home to a variety of bird and macroinvertebrates species. All of the ponds studied follow a structure similar to that of 1.2.2.3, where the ponds contain between 1 and 4 inlets discharging surface water from a variety of sites including agricultural, retail and residential developments and 1 outlet pipe which discharges to a small watercourse i.e. burn (Figure 2.3).

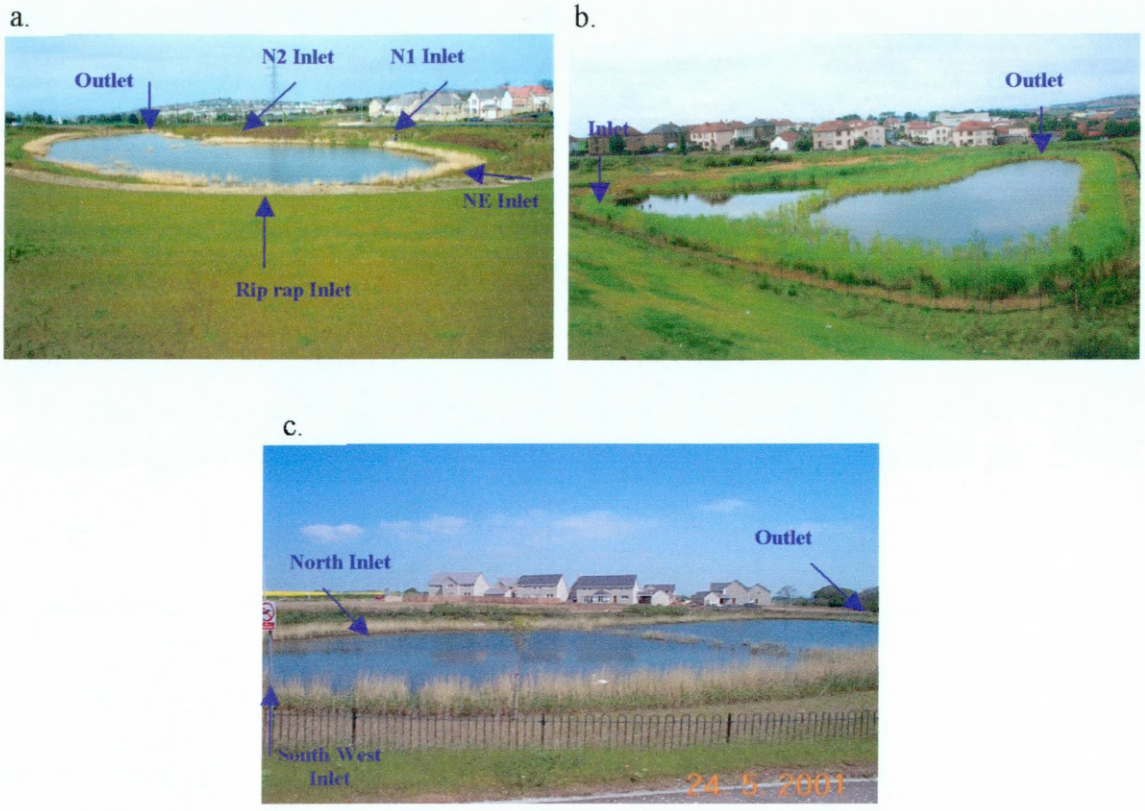


Figure 2.3 a) view of Linburn pond showing the inlets and outlet with newly developed housing constructed to the North of the pond; b) view of Halbeath pond showing the inlet and outlet with Halbeath housing estate situated to the west of the pond and c) view of Pond 7 showing the inlets and outlet with newly developed housing constructed to the north east of the pond. All of the ponds are located in the Dunfermline Eastern Expansion (DEX) Area, Duloch Park (NT1287), Dunfermline, Fife (Appendix 1).

2.1.3 Why use algae as indicators?

Algae are found in all aquatic habitats and are therefore exposed to a wide range of environmental stressors. Due to their short generation times, algal populations exhibit rapid changes in response to environmental change and xenobiotic impacts. With ease of sampling from the water column or benthos this makes algae ideal indicators to assess environmental change in aquatic ecosystems (McCormick & Cairns, 1997). More importantly, they are the primary producers of aquatic foodwebs and thus have an essential role in maintaining the stability and ecological balance of aquatic ecosystems.

2.1.3.1 Justification of selection of *C. glomerata* as an algal indicator in urban ponds

The relatively large abundance of the green filamentous macroalga *C. glomerata,* from late spring-autumn, and its dominance in all three study ponds made it the key organism of choice for study (Appendix 2A & 3). *C. glomerata* was thus used to determine the effects of biotic and abiotic change in urban ponds. It is therefore classed as a major primary producer and food web component in all three ponds.

2.1.3.2 Algal distribution

After extensive field studies to the pond sites and communication with field ecologists (Professor W Block, British Antarctic Survey) and a biological statistician (Professor Harry Staines, SIMBIOS, University of Abertay) the distribution of the macroalga *C. glomerata* at the pond fringes was assessed using a technique first developed and utilised in the field of ecology, namely quadrat analysis (Appendix 3). This is the first time this technique has been applied in SUDS systems and is used to help quantify the percentage of *C. glomerata* growth at the pond fringes. This method provides a useful quantitatively based insight into 'hot spot' areas of algal proliferation, showing accumulation/absence of algae, which can then be related to sediment quality, plant growth, seasonality and xenobiotic impacts. Data collected from this study is used to provide a clear indication of *C. glomerata* growth and population dynamics within each pond over the three-year period of the project and is therefore an invaluable tool, which will be applied to understanding the ever-changing pond ecosystem and the use of algae as a potential phytoremediator for polluted aquatic systems. In addition, the quadrat information gathered from this study has important potential applications in improving the management strategies of urban ponds.

2.1.3.3 Chlorophyll analysis in urban ponds

Chlorophylls are the primary photosynthetic pigments involved in light absorption and photochemistry in higher plants, algae and photosynthetic bacteria. Chlorophyll *a* can be found in all photosynthetic algae and chlorophyll *b*, an accessory pigment and functions as a light-harvesting pigment transferring absorbed light energy to chlorophyll *a* (Lee, 1999; Meeks, 1974). Chlorophyll determination of water samples provides an indirect measure of algal biomass and is an indicator of the trophic status of a water body (Chapman, 1996). Determination of chlorophyll *a/b* ratios in the chloroplasts of freshwater algae also provides an indication of the environment in which the algae are exposed with respect to light quality and intensity. The chlorophyll *a/b* ratio in higher plants and freshwater green algae generally range from 2:1 to 3:1 (Strain *et al.*, 1971; Kirk, 1983) and as the light intensity during growth decreases, the content of their photosynthetic pigments increases and vice-versa (normally two- to five-fold increases are observed) (Kirk, 1983; Meeks, 1974). Chlorophyll *b* is involved in the initial harvesting of light energy and it increases in plants subjected to low light conditions (Sze, 1998). However, in certain strains of algae grown under culture conditions e.g. *Chlorella vanniella*, increasing light intensity can increase the chl *a/b* ratio (Reger and Krauss, 1970). Thus, deviations from the normal chlorophyll *a/b* ratio of 2.6 in *C. glomerata* (Larkum & Barrett, 1983) can indicate exposure to 'potentially' stressful conditions in the pond system related to fluctuation in light intensity and penetration. This for example, can be linked to pond turbidity and health.

2.1.4 Water quality

The monitoring of pond water quality is an important aspect of the study as it provides pollutant load data and also indicates the state of pond health to which the algae are exposed. The water quality parameters studied follow the general sanitation analyses routinely undertaken by water authorities and monitoring waterbodies such as Scottish Environment Protection Agency (SEPA). These include the parameters pH, conductivity, suspended solids, ammoniacal nitrogen, total oxidised nitrogen (TON), and orthophosphate and chloride analyses (1.4.1 – 1.4.5).

2.1.5 Sediment quality

A SUDS research consortium comprising of five Scottish universities was set up in 1999 to study the applicability, acceptability and effectiveness of SUDS systems for new developments. The research focuses mainly in the area of engineering and hydrology, however sediment quality and the use of algae as indicators of aquatic ecosystem change in SUDS ponds have also been included in the overall SUDS research programme. The SUDS pond sediment project is designed to establish the identity and quantity of pollutants within the pond sediments over a three-year period and is carried out by Dr Kate Heal and her team at the University of Edinburgh. The sediment data collected and analysed by the University of Edinburgh team, can support and correlate with data highlighted in this chapter and project. The collaboration between the sediment and algal projects can lead to an increased understanding of the biological interactions within SUDS ponds and to the development of improved pond management strategies.

2.1.6 Pond surveys

Qualitative surveys developed by researchers within the SUDS consortium are used routinely in monitoring programmes to assess and obtain observational information on SUDS systems. The pond specific surveys were incorporated into this research project to provide an observational assessment of SUDS ponds throughout the sampling season and to critically evaluate the usefulness and interpretation of survey data for a monitoring programme. Observations were scored on a survey tick sheet (Surveys 1A – 3C) and include comments on inlet/outlet discharges, sediment build up at inlets, colour of sample water, bank erosion, algal distribution at particular sites and the presence/absence of wildlife (birds/insects).

2.2 Materials and Methods

2.2.1 Chlorophyll *a* analysis (24 hour acetone method)

Water samples (~2L) were removed from discharging inlet and outlet pipes and from areas outwith the inlets and outlets to fully investigate pond water quality and to determine chlorophyll concentrations for estimating pond algal biomass. At all times, care was taken to prevent sediment from entering the samples. Samples were kept cool and in the dark to prevent photooxidation whilst sampling and were processed within 24 hrs of collection.

Water samples of a known volume were filtered through a Grade 1 Whatman filter using a vacuum pump and the filtrate discarded. After filtration was complete, the air was drawn through the filter for approximately 5min to reduce the residual water content of the filter. The filter paper was removed, rolled carefully, transferred to a test

tube containing 14ml of 90% (v/v) acetone and agitated with forceps to ensure complete contact of the paper with the acetone. The tube was stoppered and placed in a dark refrigerator (4°C) for 24hrs. The tube was then centrifuged (Jouan A14) at 3,5000rpm for 7min and the clear solution containing the acetone decanted to a quartz cuvette and read at 665nm and 750nm using 90% (v/v) acetone as a reference blank. The absorbance value at A_{665}nm should fall within the range of 0.050 to 0.700 units and at A_{750}nm should not exceed 0.005 units per 10mm of cell wavelength. The absorbance value obtained at A_{750}nm was extracted from A_{665}nm and the resulting value represents A in the equation below (Standing Committee of Analysts, 1983).

The chlorophyll *a* content of samples were calculated using the equation below:

$$\text{Chlorophyll } a \ (\mu g.\text{chl } a.l^{-1}) = \frac{11.9 \times A \times v}{d \times V}$$

Where A = absorbance (A_{750}nm – A_{665}nm)

v = volume of acetone in ml

V = volume of initial filtered sample in litres and

d = cell pathlength in cm.

2.2.2. Chlorophyll *a/b* analysis

Samples of *C. glomerata* collected from specific sites at Halbeath pond during spring-summer 2001 were removed from the –80°C freezer (Holden, 1976) in January 2002, thawed, blotted dry and weighed into ~500mg aliquots. The samples were chopped into smaller sections added to a mortar and pestle (Hellebust & Craigie, 1978) and two applications of liquid nitrogen were applied whilst grinding (Chapman, 1988). 10ml of methanol were added for 1min and the aqueous/methanol layer removed using a pipette. The algal suspension was transferred to a McCartney bottle containing 14ml 90% (v/v)

acetone (Sigma) and a pinch of $MgCO_3$ and was refrigerated in the dark at 4°C for 24hrs. To remove the residual $MgCO_3$, the suspension was transferred to centrifuge tubes, spun at 3500rpm for 7min and the clear solution containing the acetone decanted to a quartz cuvette and read at 664nm and 647nm using 90% acetone as a reference blank (Holden, 1976; Sterman, 1988). The absorbance values obtained at A_{664}nm and A_{647}nm were inserted into the equation below to obtain the ratio of chlorophyll *a*/*b* for each sample (Jeffrey & Humphrey, 1975).

$$\text{Chlorophyll } a \text{ } (\mu g.chl.ml^{-1}) = 11.93\ (A_{664}nm) - 1.93\ (A_{647}nm)$$

$$\text{Chlorophyll } b \text{ } (\mu g.chl.ml^{-1}) = 20.36\ (A_{647}nm) - 5.50\ (A_{664}nm)$$

2.2.3 Water quality analysis

Water samples (~1L) were taken from discharging inlets and outlet pipes, transported to SEPA laboratories on the day of sampling and a general sanitation analysis performed within 72 hrs. Analysis of pH, conductivity, suspended solids, ammoniacal nitrogen, total oxidised nitrogen (TON), orthophosphate and chloride concentrations were carried out using the methods as described by the Standard Methods for the Examination of Water and Wastewater (Eaton *et al.*, 1992). Periodically, a small number of water samples were analysed for biological oxygen demand (BOD) (this data was omitted from the results section due to the small number of data points).

2.2.4 Quadrat analyses

C. glomerata populations were assessed every fortnight from April – September using a quadrat with dimensions of $25cm^2$. The quadrat was placed every two metres at the pond fringes from a standardised starting point and the coverage of *C. glomerata* within

the quadrat scored as a percentage value in the range of 0 –20%, 20 – 40%, 40 – 70%, 70 – 90% and 90 – 100%. Co-ordinates of the pond sampling points were inputted into MapInfo (Microsoft version 5.5) and the percentage values of algal coverage corresponding to the relevant co-ordinates were added to produce maps which quantatively show the distribution and abundance of *C. glomerata* in each pond over a three year period.

2.2.5 Sediment surveys

Pond sediment surveys carried out by Dr Kate Heal and her team at the University of Edinburgh were used to support and correlate with data collected in this chapter (2.1.5). Sediment cores were removed from specific transects (Appendix 4) representative of each pond including pond inlets and outlets on a yearly basis (1999 – 2001) and analysed for cadmium, chromium, copper, iron, lead, nickel, zinc, total nitrogen and total phosphorous using standardised sediment protocols (Heal, 2002) (Appendix 4).

2.2.6 Pond surveys and site visits

Site visits were made to each pond on a fortnightly basis from March – September, 2000 – 2002. Surveys carried out on the selected ponds include the collection of water samples at selected pond areas (Figures 2.4 – 2.10), quadrat surveys (Figures 2.11 – 2.19) and the completion of pond specific observation surveys (Surveys 1A–3C).

2.3 Results

2.3.1 Chlorophyll *a* analysis

Chlorophyll *a* analysis was performed on water samples collected from pond inlets and outlets during the sampling season April – September 2001 and April – June 2002. Chlorophyll *a* levels for all water samples collected during this period were either zero, or at the limits of assay detection.

2.3.2 Chlorophyll determination of *C. glomerata*

Table 2.1 shows that *C. glomerata* chlorophyll *a/b* ratios can vary from 2.0 to 3.3 depending on sampling month and site of collection. The majority of the samples collected fell below the normal chl *a/b* ratio of 2.6 for *C. glomerata* with the exception of Halbeath pond June South and September West samples that show increased chl *a/b* ratios of 3.3 and 2.9 respectively.

Table 2.1 Summary of *C. glomerata* chlorophyll *a/b* ratios after extraction (n = 3) from Halbeath pond.

Month	Site	Chl *a*	Chl *b*	Chl *a*: Chl *b* ($\mu g.chl.ml.500mg\ freshwt^{-1}$)
June 2001	Outlet	13.5	5.9	2.3
	East	8.3	3.5	2.4
	North	8.6	3.5	2.5
	Inlet	13.1	4	3.3
July 2001	Outlet	12.0	5.5	2.2
	East	10.2	4.4	2.3
	North	12.7	5.9	2.2
	Inlet	11.0	5.0	2.2
August 2001	Outlet	12.8	6.3	2.0
	East	9.6	4.4	2.2
	North	7.7	3.4	2.3
	Inlet	9.7	3.9	2.5
September 2001	Outlet	9.7	3.4	2.9
	East	9.6	4.6	2.1

2.3.3 Pond Trophic Status

Table 2.2 illustrates the trophic status of water quality samples collected from pond inlets and outlets during each sampling month over a period of 2 years. The orthophosphate values obtained from the ponds are compared to the total phosphorous classification limits set by SEPA for Scottish Freshwater Lakes (SEPA, 1997). The results show large variations in the trophic range between ponds and sampling month and site (1.4.5.1). The majority of Linburn pond samples fall into the hypertrophic range (> $100\mu g.l^{-1}$ P) with Halbeath pond samples in the oligotrophic-mesotrophic category (10 - $35\mu g.l^{-1}$ P) and Pond 7 in the mesotrophic-eutrophic range (35 - $100\mu g.l^{-1}$ P) (SEPA, 1997). Samples were not collected from Dec 2000 – Feb 2001 and absent values outwith this period indicate no discharge at the inlets or outlets.

Table 2.2 Monthly trophic status of water samples (n = 2) taken from pond inlets and outlets during June 2000 to 2002.

Month	Lin RR Inlet	Lin NE Inlet	Lin N1 Inlet	Lin N2 Inlet	Lin Outlet	Hal Inlet	Hal Outlet	P7 SW Inlet	P7 N Inlet	P7 Outlet
Jun 2000	ME	ME	H	H	ME	ME	OM	ME	OM	-
Jul 2000	ME	OM	OM	OM	OM	OM	OM	OM	OM	OM
Aug 2000	ME	OM	OM	OM	OM	OM	OM	OM	OM	-
Sept 2000	H	OM	ME	ME	OM	OM	OM	ME	OM	OM
Oct 2000	H	OM	ME	ME	ME	OM	OM	ME	OM	OM
Nov 2000	ME	OM	OM	ME	ME	OM	OM	ME	OM	ME
Dec 2000	-	-	-	-	-	-	-	-	-	-
Jan 2001	-	-	-	-	-	-	-	-	-	-
Feb 2001	-	-	-	-	-	-	-	-	-	-
Mar 2001	ME	OM	H	ME	H	OM	OM	ME	OM	OM
Apr 2001	ME	OM	H	H	H	OM	ME	ME	OM	OM
May 2001	ME	OM	H	H	H	ME	OM	ME	OM	-
Jun 2001	H	ME	H	-	H	H	OM	H	-	-
Jul 2001	H	H	H	H	H	H	H	H	-	OM
Aug 2001	H	H	ME	-	ME	H	ME	H	H	-
Sept 2001	H	H	H	H	H	ME	ME	H	H	OM
Oct 2001	H	H	H	H	ME	ME	OM	H	ME	-
Nov 2001	H	H	ME	-	H	H	ME	H	ME	ME
Dec 2001	H	ME	H	-	H	ME	ME	H	ME	ME
Jan 2002	H	-	H	-	H	OM	OM	H	ME	ME
Feb 2002	H	ME	H	-	H	H	-	H	H	H
Mar 2002	ME	ME	H	-	H	OM	OM	ME	ME	OM
Apr 2002	ME	-	-	-	H	OM	-	ME	ME	-
May 2002	ME	OM	ME	-	ME	OM	ME	ME	ME	OM
Jun 2002	ME	ME	OM	-	ME	OM	OM	ME	ME	-

Table Key:

Lin = Linburn pond
Hal = Halbeath pond
P7 = Pond 7
RR = Rip Rap Inlet
NE = North East Inlet
N1 = North 1 Inlet
N2 = North 2 Inlet
SW = South West Inlet

OM = oligotrophic-mesotrophic range (10-35$\mu g.l^{-1}$ P) (low biological activity)
ME = mesotrophic-eutrophic range (35-100$\mu g.l^{-1}$ P) (balanced system)
H = hypertrophic range (> 100$\mu g.l^{-1}$ P) (high biological activity)
- = no discharge at inlet/outlet

2.3.4 Water quality

Water quality data (n = 1) collected during a two-year period (June 2000 – June 2002) shows marked variation between ponds but also between inlet and outlet samples (samples were not collected from Dec 2000 – Feb 2001 as they are out of the algal growth season and absent values out with this period indicate no discharge at the inlets or outlets).

The pH levels for all ponds studied generally fell within the normal range for natural waters of 6.0 to 8.5, with the exception of a minority of Linburn pond and Pond 7 samples which had a pH > 8.5 and may be due to cement inputs from construction sites (Figure 2.4)

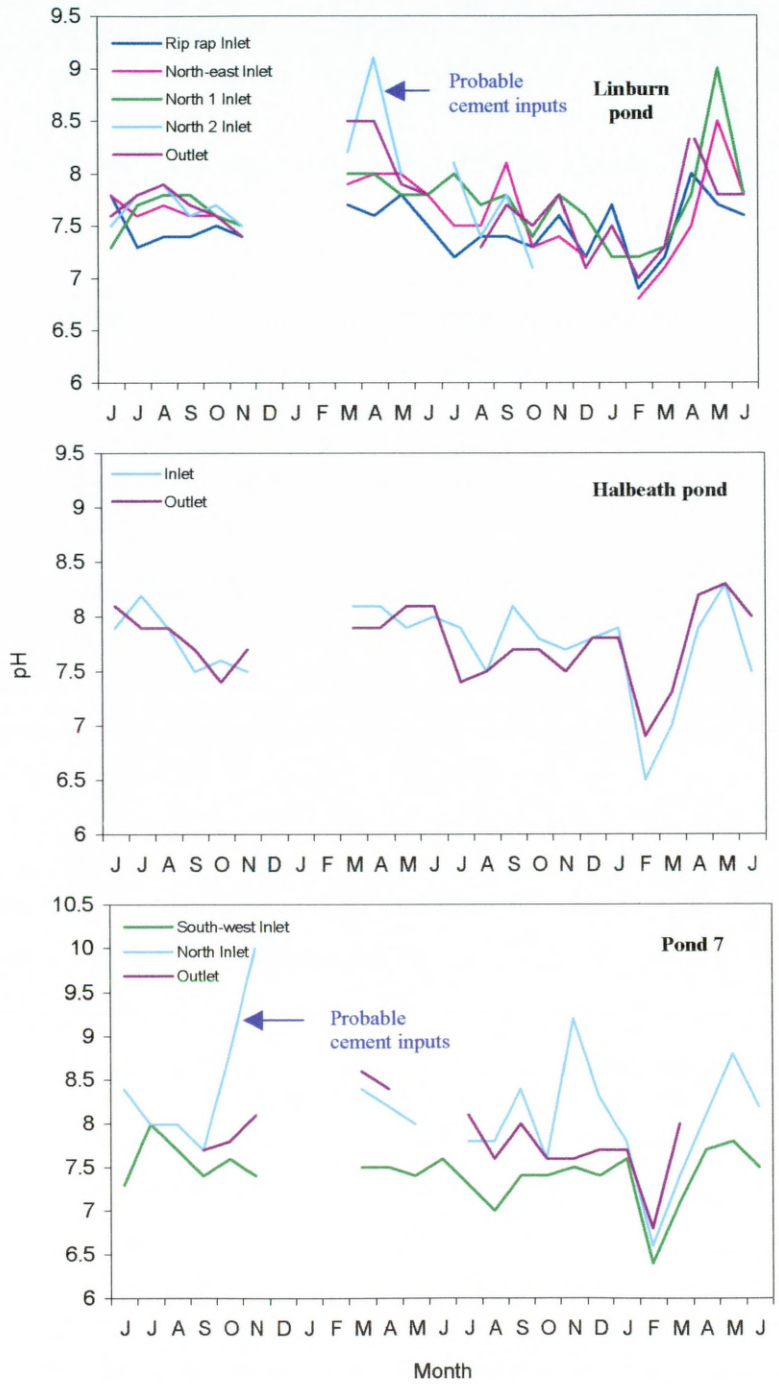


Figure 2.4 Change in pH from June 2000 – 2002 in Linburn pond, Halbeath pond and Pond 7 (n = 2/month). [missing data – no analysis performed outwith algal growth season or due to lack of flow].

Conductivity levels in Figure 2.5 show large variations between monthly samples and in some cases this may be due to salt applications on road surfaces during the winter and early spring months (as illustrated in Figure 2.5) (Scott, 1976). Generally, conductivity levels for the samples were within the normal conductivity freshwater range of 10 to 1000 $\mu S.cm^{-1}$ (Chapman, 1996).

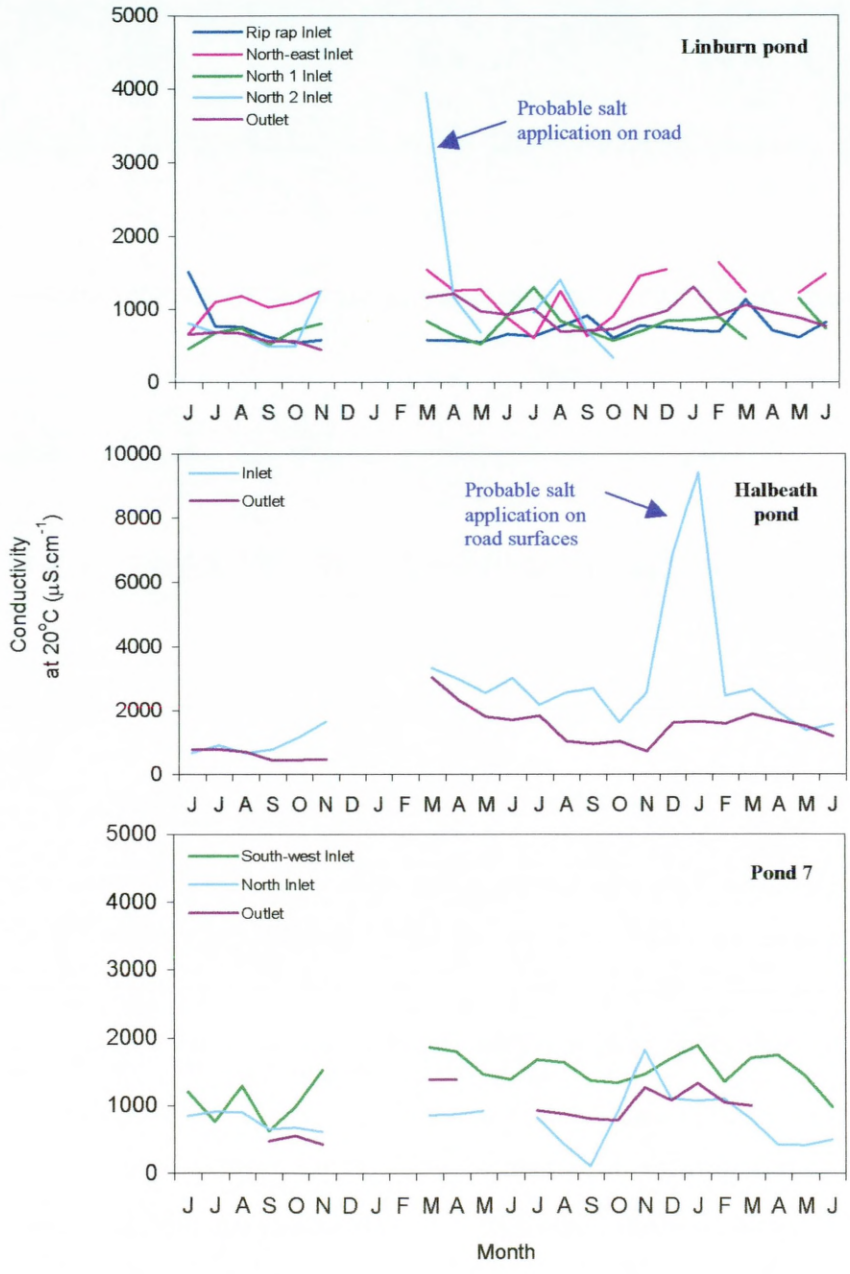


Figure 2.5 Change in Conductivity at 20°C ($\mu S.cm^{-1}$) from June 2000 – 2002 in Linburn pond, Halbeath pond and Pond 7 (n = 2/month). (*Note the scale on the conductivity axis changes between graphs) [missing data – no analysis performed outwith algal growth season or due to lack of flow].

Figure 2.6 shows large variations in monthly suspended solid data due to high rainfall and floods within the catchment prior to sampling or during sampling. Samples that display high suspended solid concentrations > 100 $mg.l^{-1}$ are likely to be associated with construction site solid inputs and soil erosion (as indicated on the graphs). Generally, the suspended solid concentrations < 50 $mg.l^{-1}$, which at these concentrations are unlikely to cause adverse effects to fish health (Alabaster, 1980).

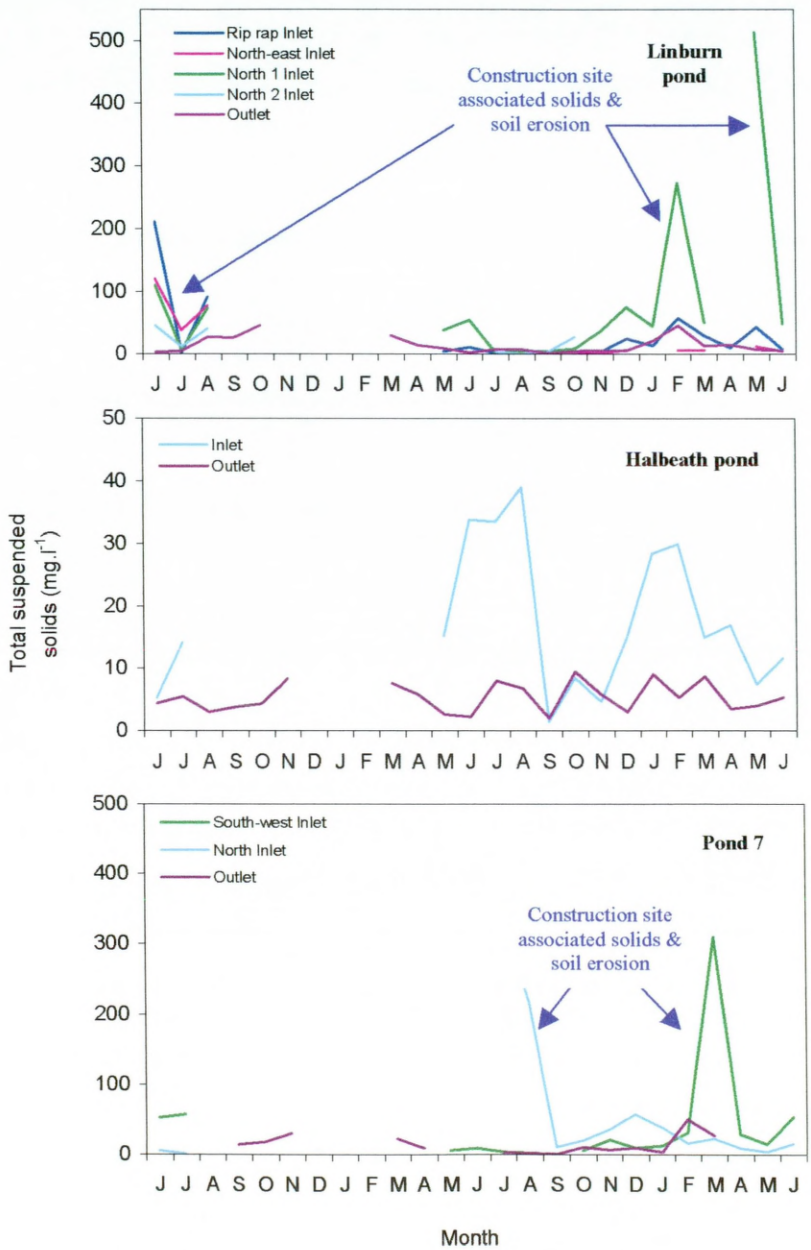


Figure 2.6 Change in Total Suspended Solid concentrations ($mg.l^{-1}$) from June 2000 – 2002 in Linburn pond, Halbeath pond and Pond 7 (n = 2/month). (*Note the scale on the total suspended solid axis changes between graphs) [missing data – no analysis performed outwith algal growth season or due to lack of flow].

Ammoniacal nitrogen (NH_3-N) concentrations for Linburn pond, Halbeath pond and Pond 7 water samples were < 0.5 $mg.l^{-1}$ with the exception of a minority of Linburn pond samples that contained excessive ammoniacal nitrogen concentrations of > 10 $mg.l^{-1}$ (as indicated on the graphs) (Figure 2.7). Typically, surface waters contain an ammoniacal nitrogen concentration of 2 – 3 $mg.l^{-1}$ where elevated levels generally indicate organic pollution (Chapman, 1996).

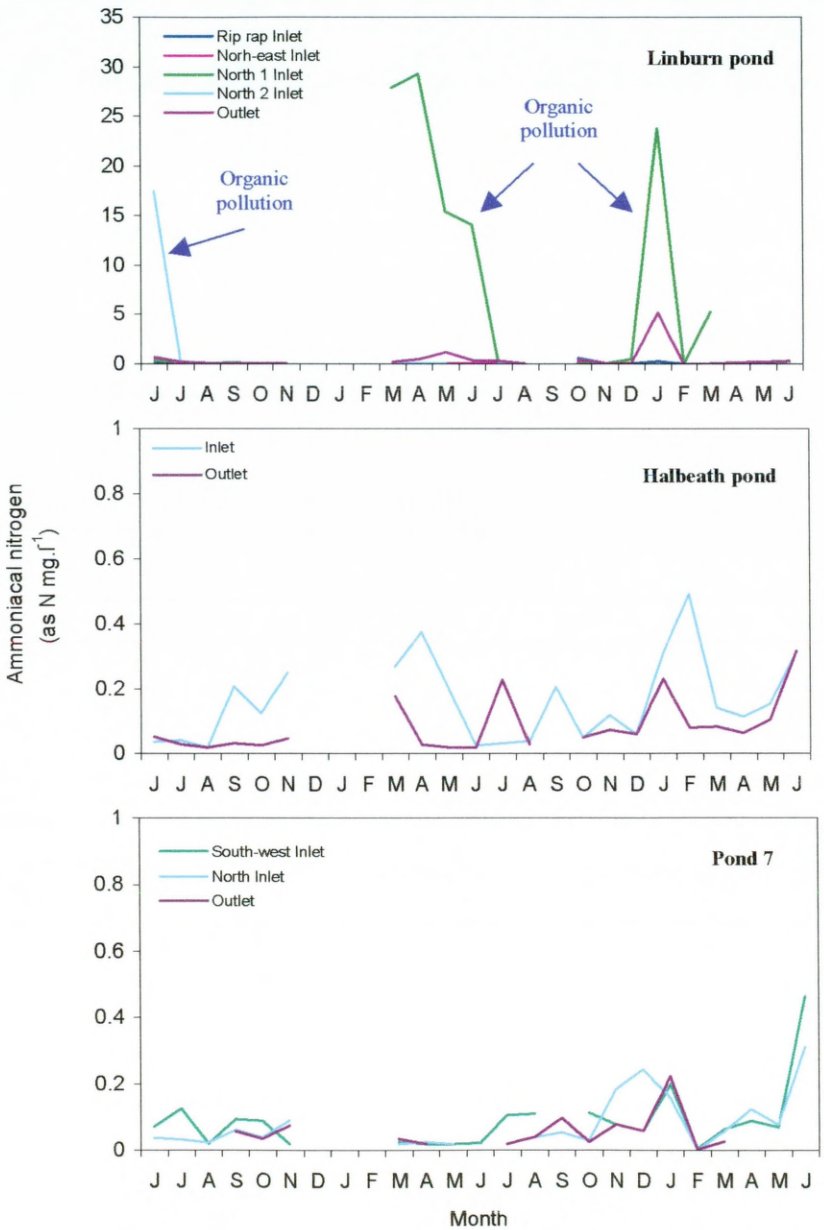


Figure 2.7 Change in Ammoniacal Nitrogen concentrations (as N $mg.l^{-1}$) from June 2000 – 2002 in Linburn pond, Halbeath pond and Pond 7 (n = 2/month). (*Note the scale on the ammoniacal nitrogen axis changes between graphs) [missing data – no analysis performed outwith algal growth season or due to lack of flow].

TON concentrations (NO_3-N and NO_2-N) for all pond samples are below 5mg.l^{-1} with the exception of one Linburn pond sample (June 2000) with a TON concentration of 9.2 mg.l^{-1} (Figure 2.8). Surface water concentrations in excess of 5 mg.l^{-1} usually indicate pollution from domestic sewage or fertiliser runoff (Chapman, 1996).

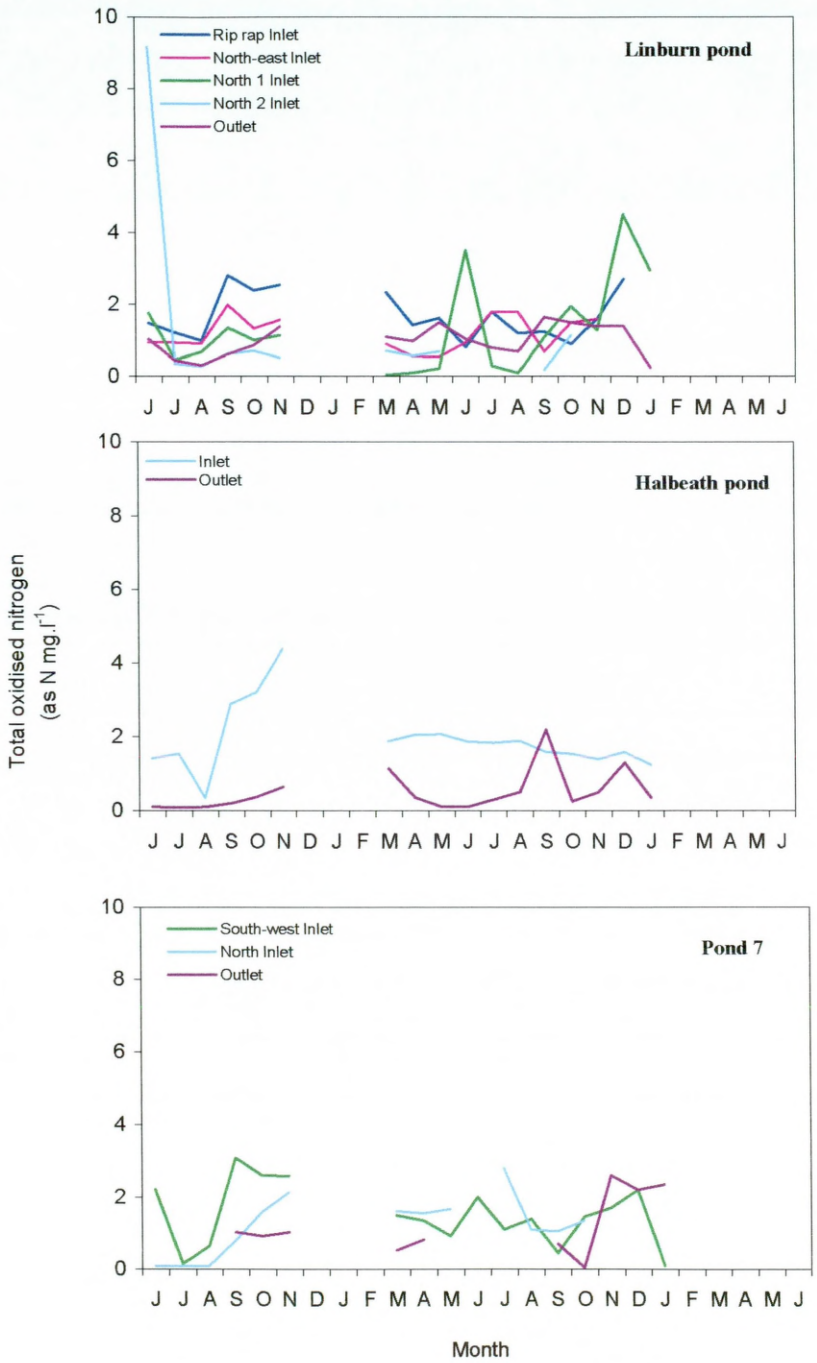


Figure 2.8 Change in Total Oxidised Nitrogen concentrations (as N mg.l^{-1}) from June 2000 – 2002 in Linburn pond, Halbeath pond and Pond 7 (n = 2/month) [missing data – no analysis performed outwith algal growth season or due to lack of flow].

Orthophosphate concentrations (PO_4-P) for the majority of pond water samples < 0.5 $mg.l^{-1}$ with the exception of a few Linburn pond and Pond 7 samples that show elevated PO_4-P concentrations > 0.5 $mg.l^{-1}$ (Figure 2.9). High PO_4-P concentrations are largely responsible for eutrophic conditions in aquatic systems but it can be reduced by plant/ algal uptake during summer and increased due to algal death/reed dieback during winter (as indicated on the graphs).

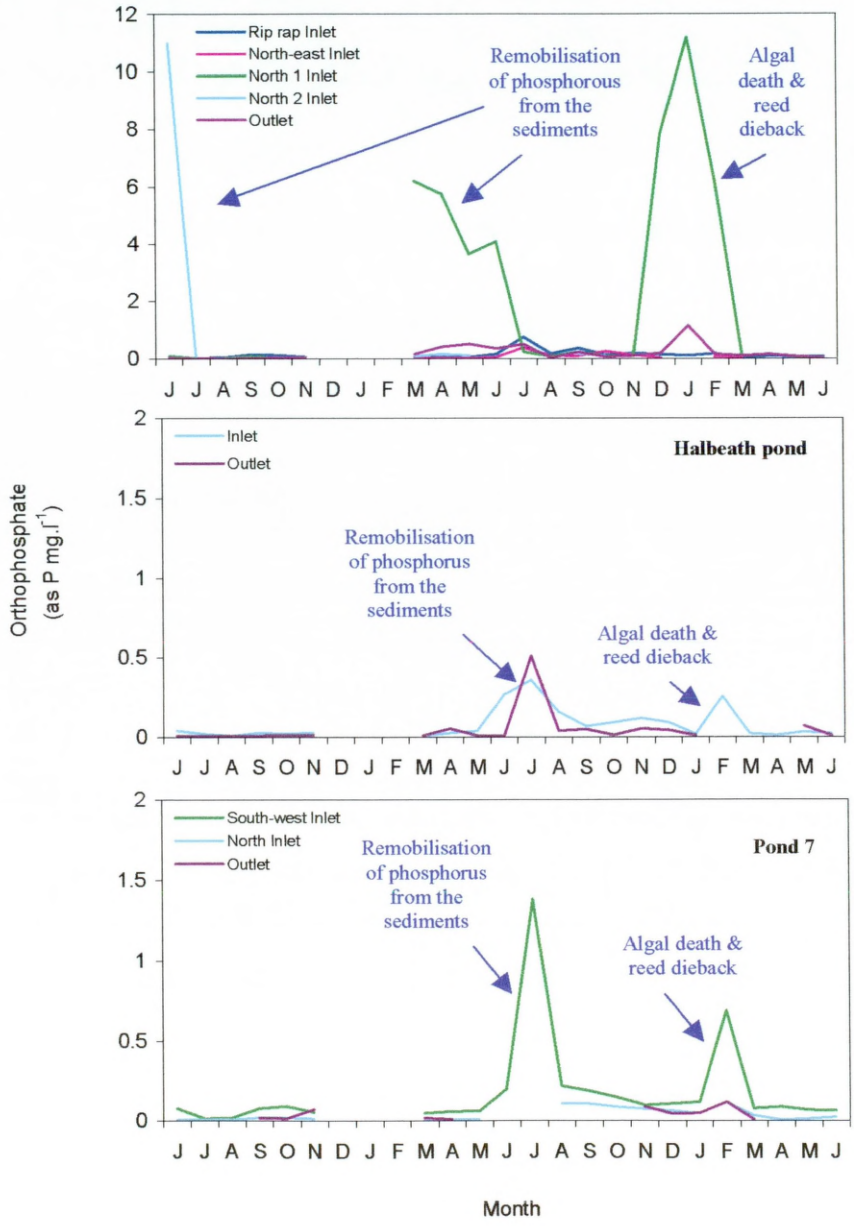


Figure 2.9 Change in Orthophosphate concentrations (as P $mg.l^{-1}$) from June 2000 – 2002 in Linburn pond, Halbeath pond and Pond 7 (n = 2/month). (*Note the scale on the orthophosphate axis changes between graphs) [missing data – no analysis performed outwith algal growth season or due to lack of flow].

Figure 2.10 shows large seasonal fluctuations in monthly chloride concentration data for each pond probably due to road salt applications during winter periods (as indicated on the graphs). Generally, samples fall below chloride concentrations of 400 $mg.l^{-1}$, which at these concentrations usually indicates pollution by humans or irrigation drains (Chapman, 1996).

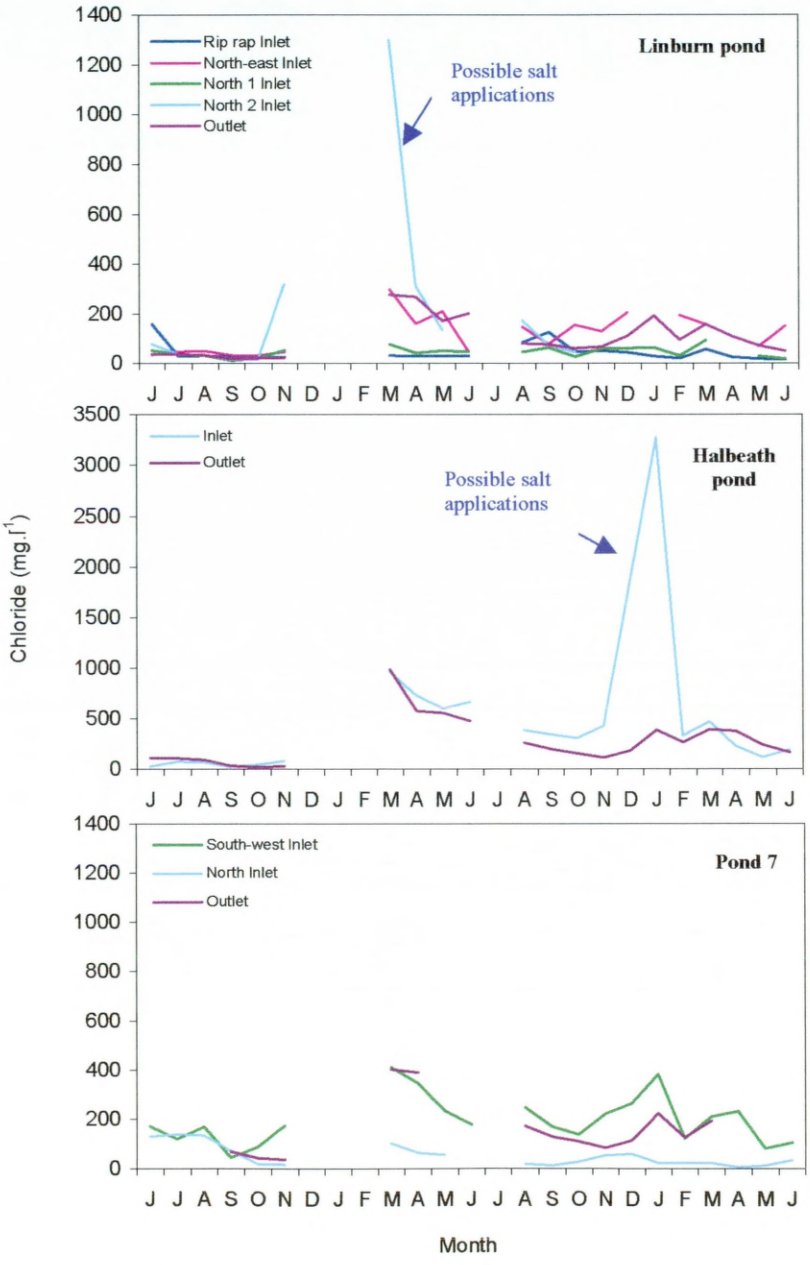


Figure 2.10 Change in Chloride concentrations ($mg.l^{-1}$) from June 2000 – 2002 in Linburn pond, Halbeath pond and Pond 7 (n = 2/month). (*Note the scale on the chloride axis changes between graphs) [missing data – no analysis performed outwith algal growth season or due to lack of flow].

2.3.5 Algal distribution

Algal distribution data demonstrated marked variation in the percentage growths of *C. glomerata* at the peak of the growing season (July), as described by Planas *et al.*, 1996, not only between ponds but also between years sampled. During 2000, there is significant algal coverage within the ponds, but the abundance steadily decline during year 2001 and 2002. The distribution of algae at selected pond locations also varies from year to year i.e. *C. glomerata* accumulation at north and south locations in Linburn pond during 2000 but only south in 2002 (Figures 2.11 & 2.13); *C. glomerata* abundance at east and west locations in Halbeath pond during 2000 but north and east in 2002 (Figures 2.14 & 2.16) and *C. glomerata* widely distributed throughout Pond 7 during 2000 but mainly located at south and east locations in 2002 (Figures 2.17 & 2.19).

2.3.6 Sediment quality

Yearly analysis of pond sediments (1999 – 2001) carried out by Dr Kate Heal and her team at the University of Edinburgh (2.1.5), indicate that certain metals will accumulate as the ponds age (Appendix 4B – 4D) (Heal, 2002). Sediment analyses of Halbeath pond, Linburn pond and Pond 7 during 2001 show increases in metal concentrations when compared with sediment data collected during 1999 (Appendix 4B & 4D). Increased concentrations of chromium, nickel and lead were detected in all three ponds during 2001, with increased zinc in both Halbeath and Linburn pond, increased cadmium in Halbeath and Pond 7 and increased iron in Linburn and Pond 7 (Appendix 4D). Only Halbeath pond showed an increase in copper concentration during 2001. All of the pond sediments analysed during 2001 decreased in both nitrogen and phosphorous concentration (Appendix 4D).

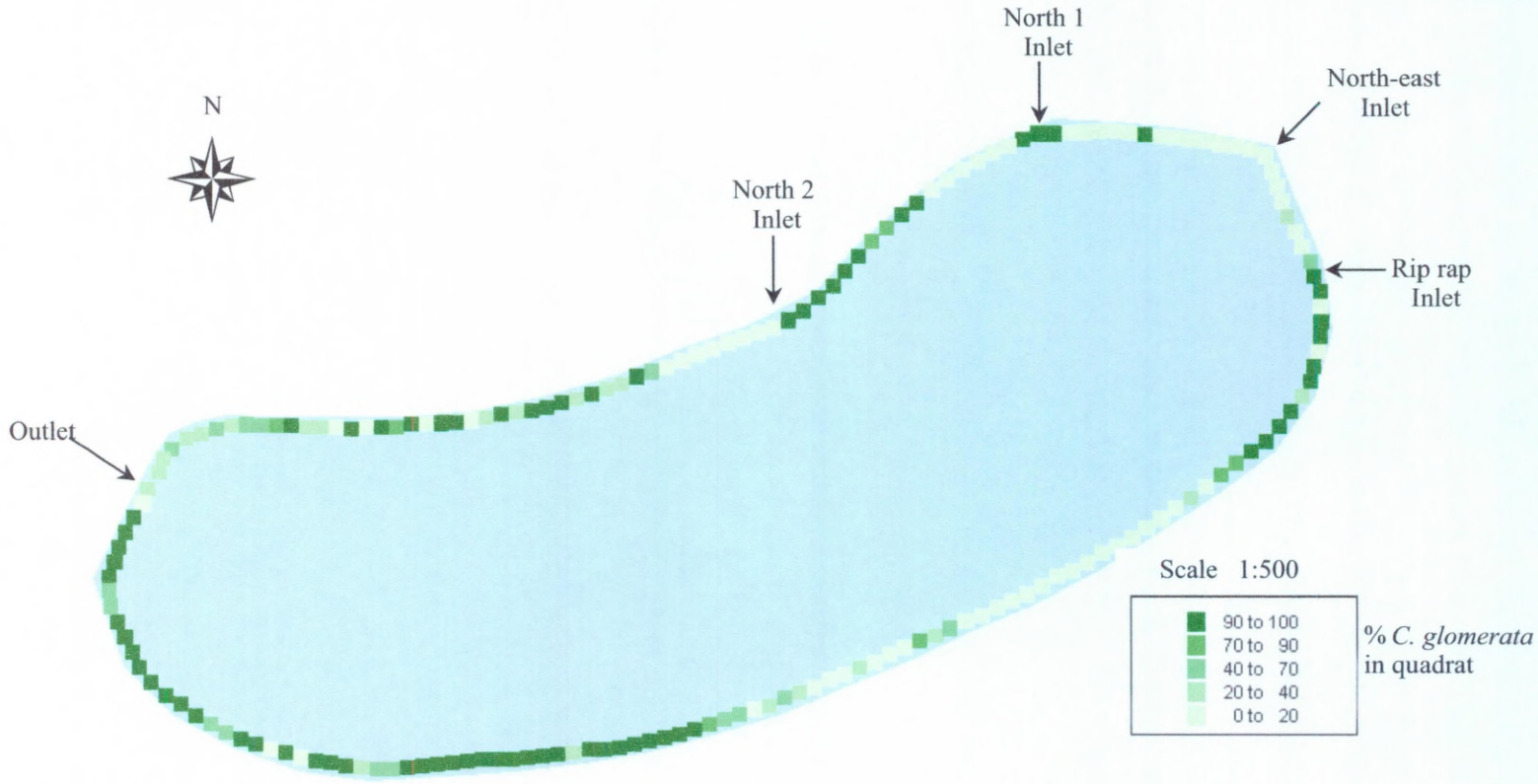


Figure 2.11 *C. glomerata* distribution Linburn pond 2000

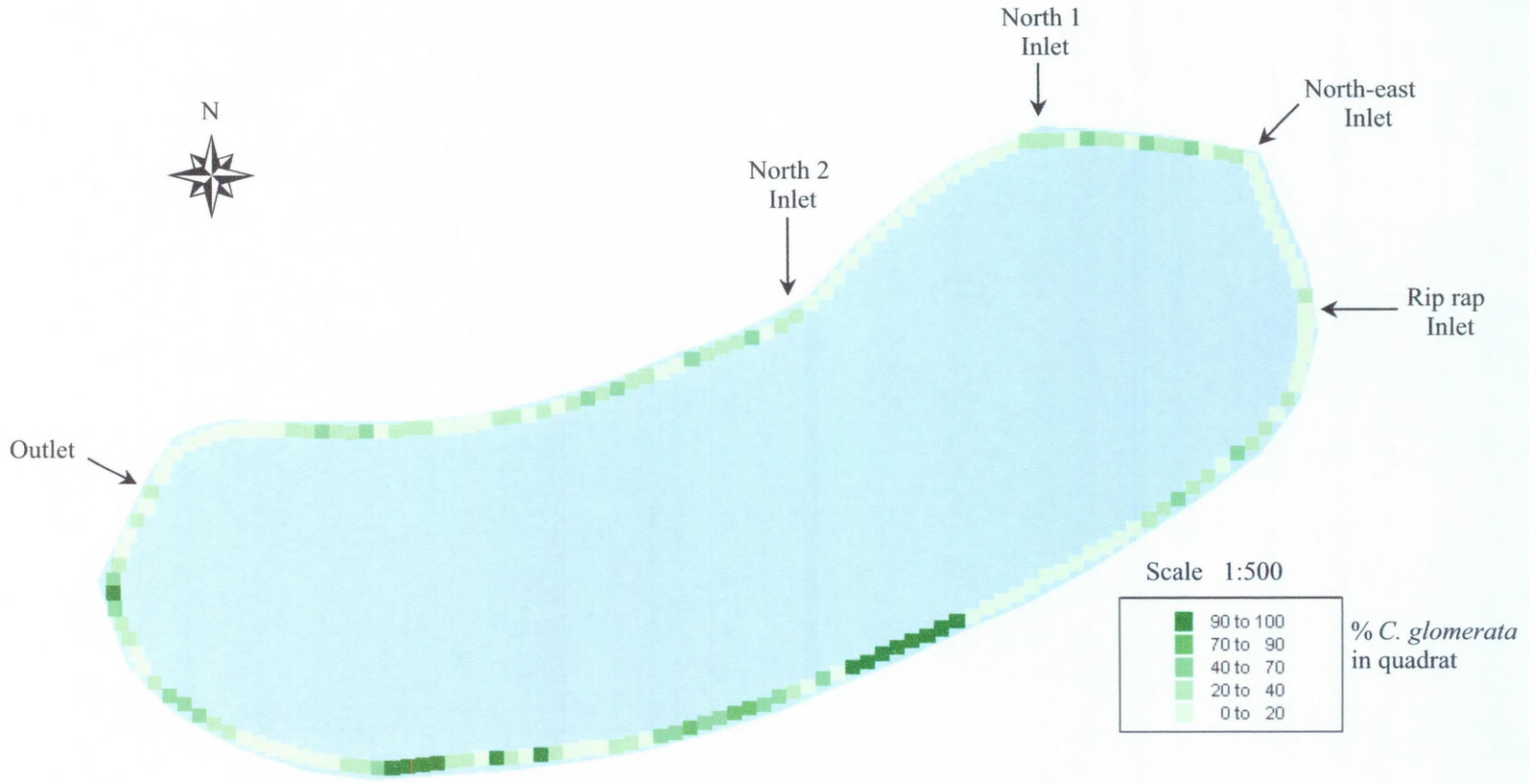


Figure 2.12 *C. glomerata* distribution Linburn pond 2001

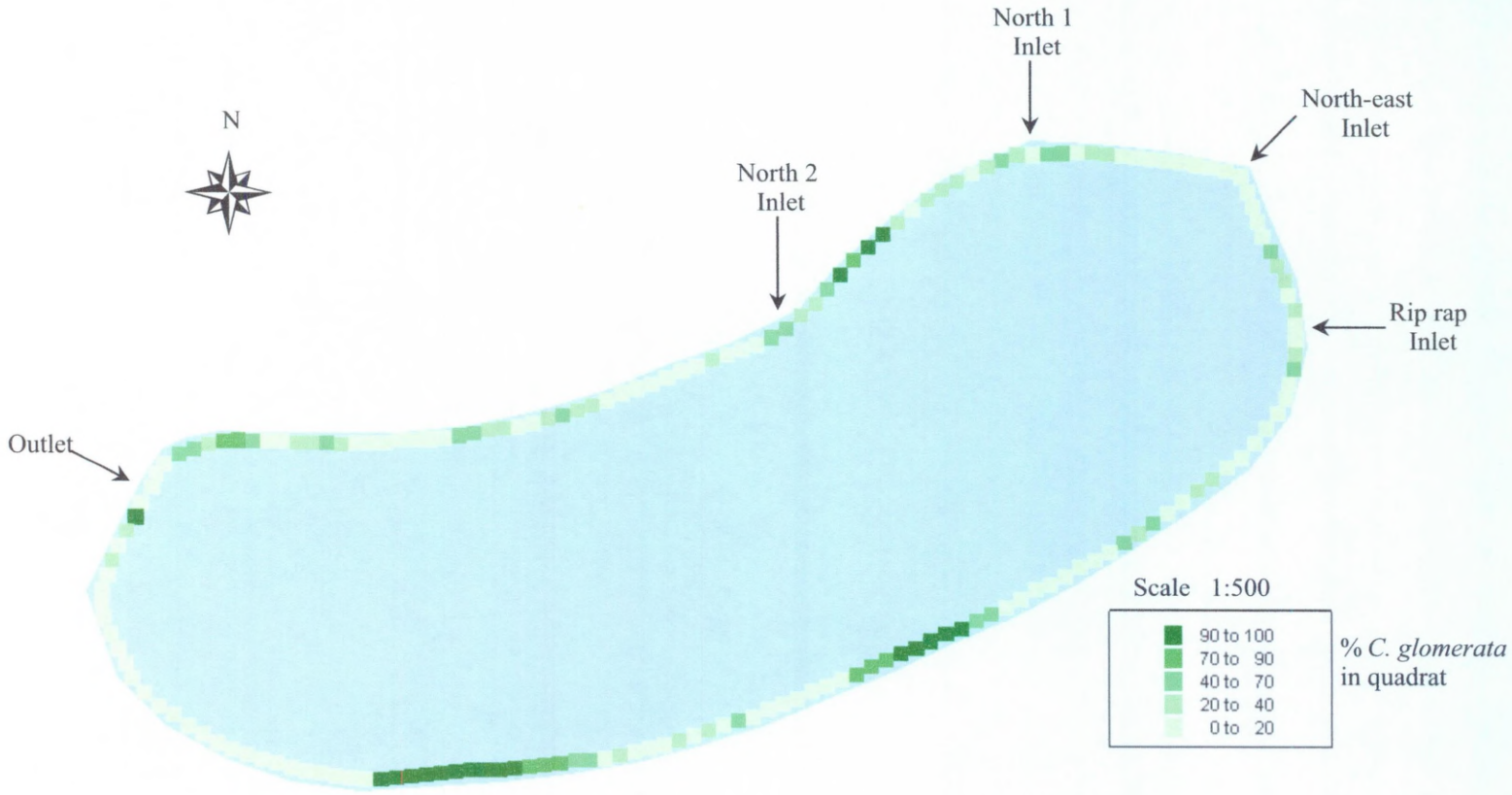


Figure 2.13 *C. glomerata* distribution Linburn pond 2002

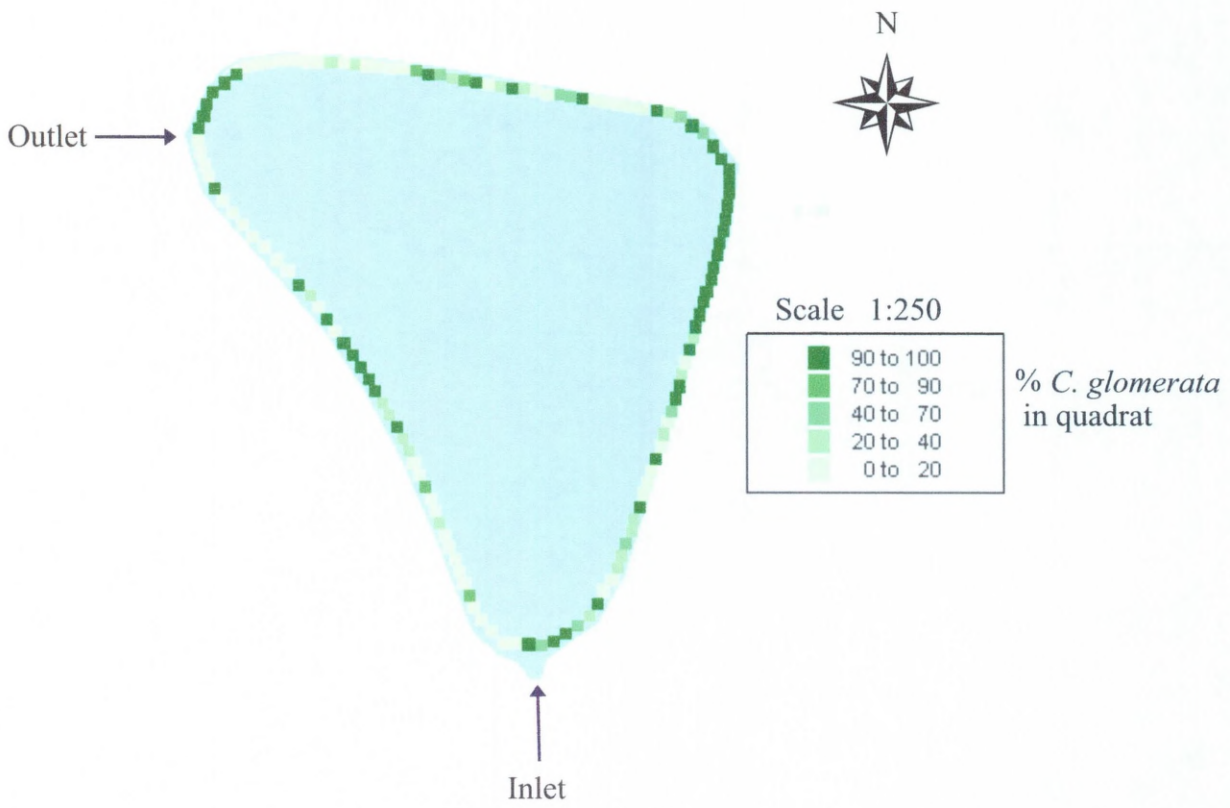


Figure 2.14 *C. glomerata* distribution Halbeath pond 2000

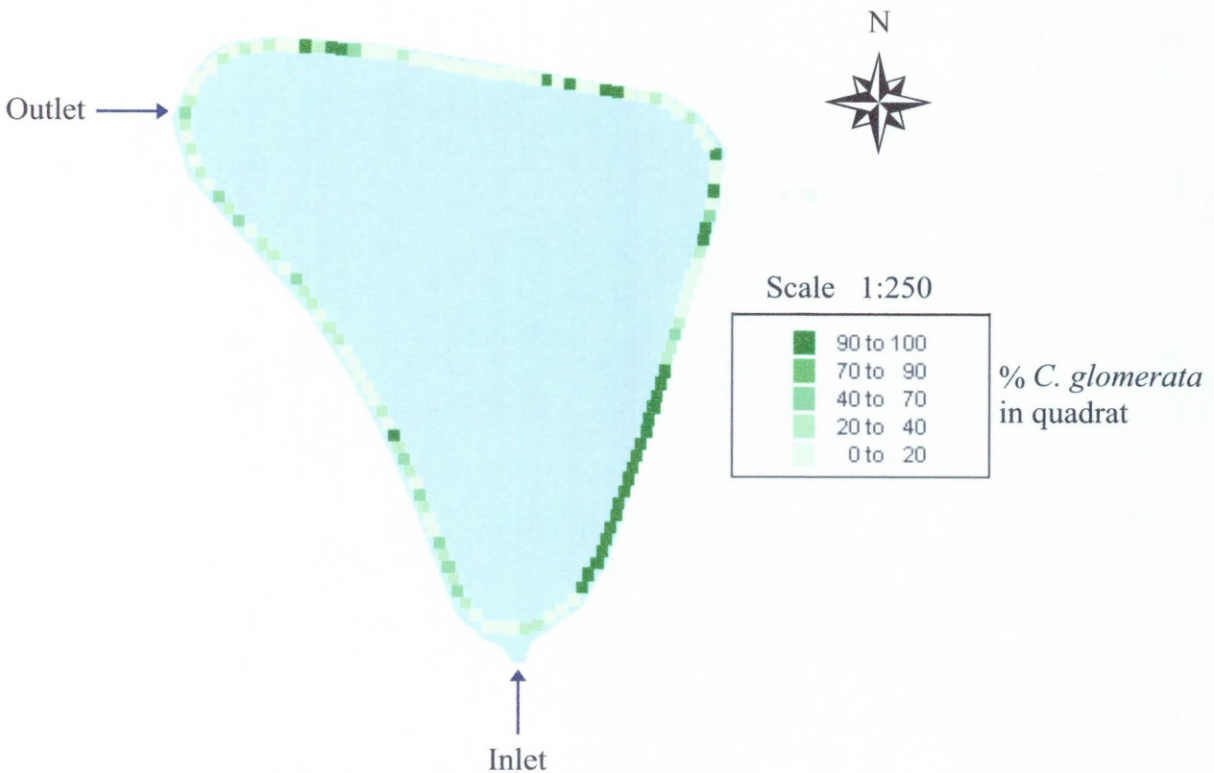


Figure 2.15 *C. glomerata* distribution Halbeath Pond 2001

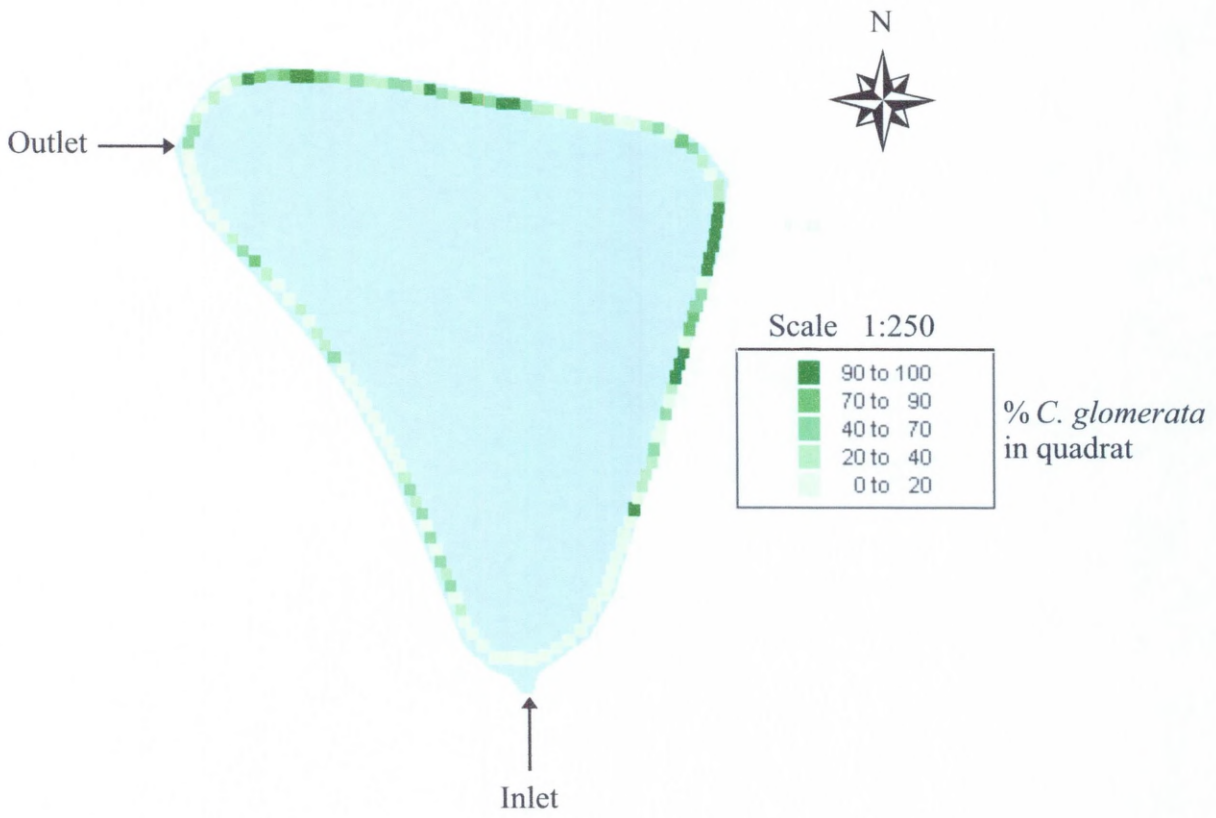


Figure 2.16 *C. glomerata* distribution Halbeath Pond 2002

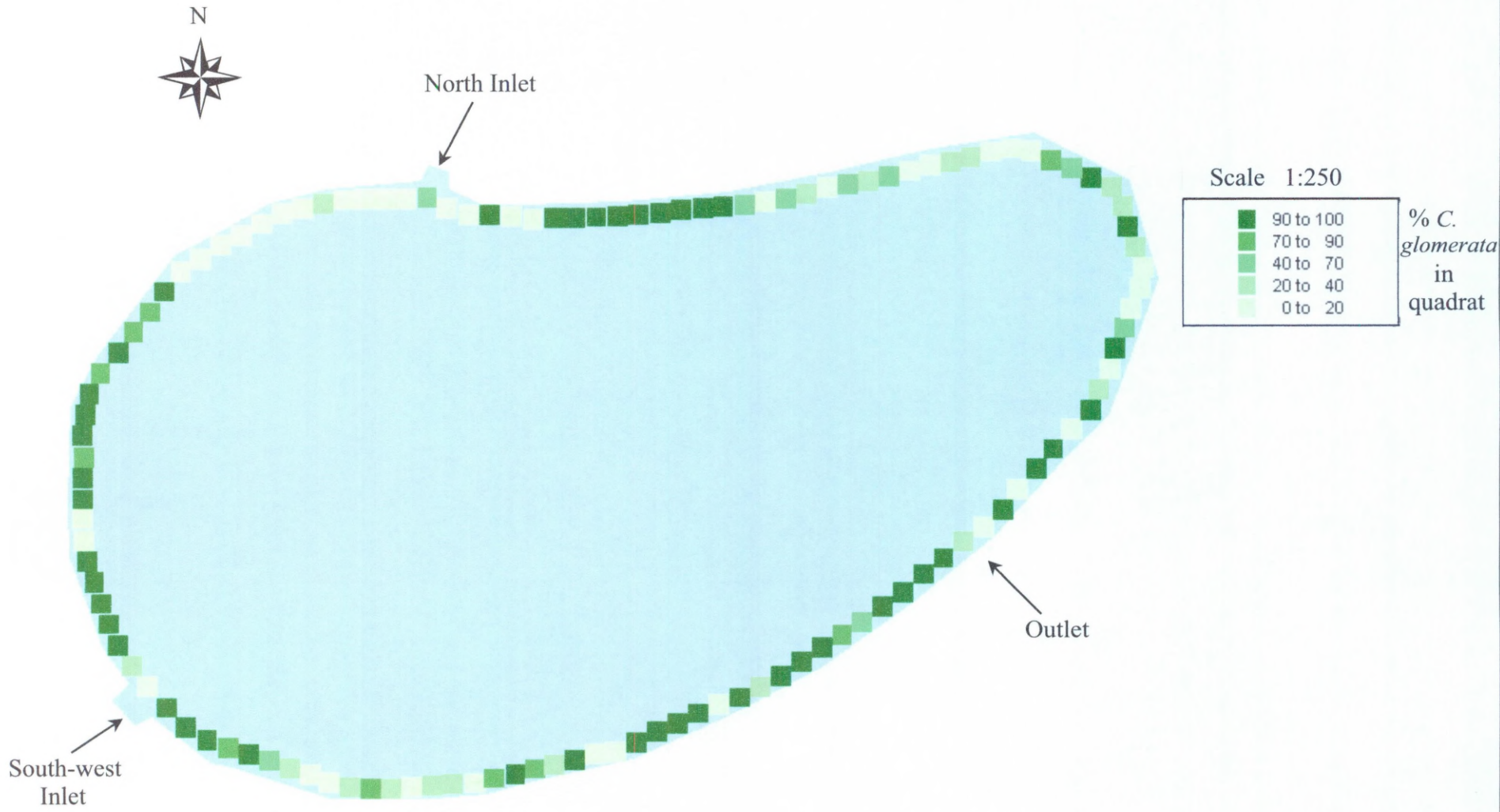


Figure 2.17 *C. glomerata* distribution Pond 7 2000

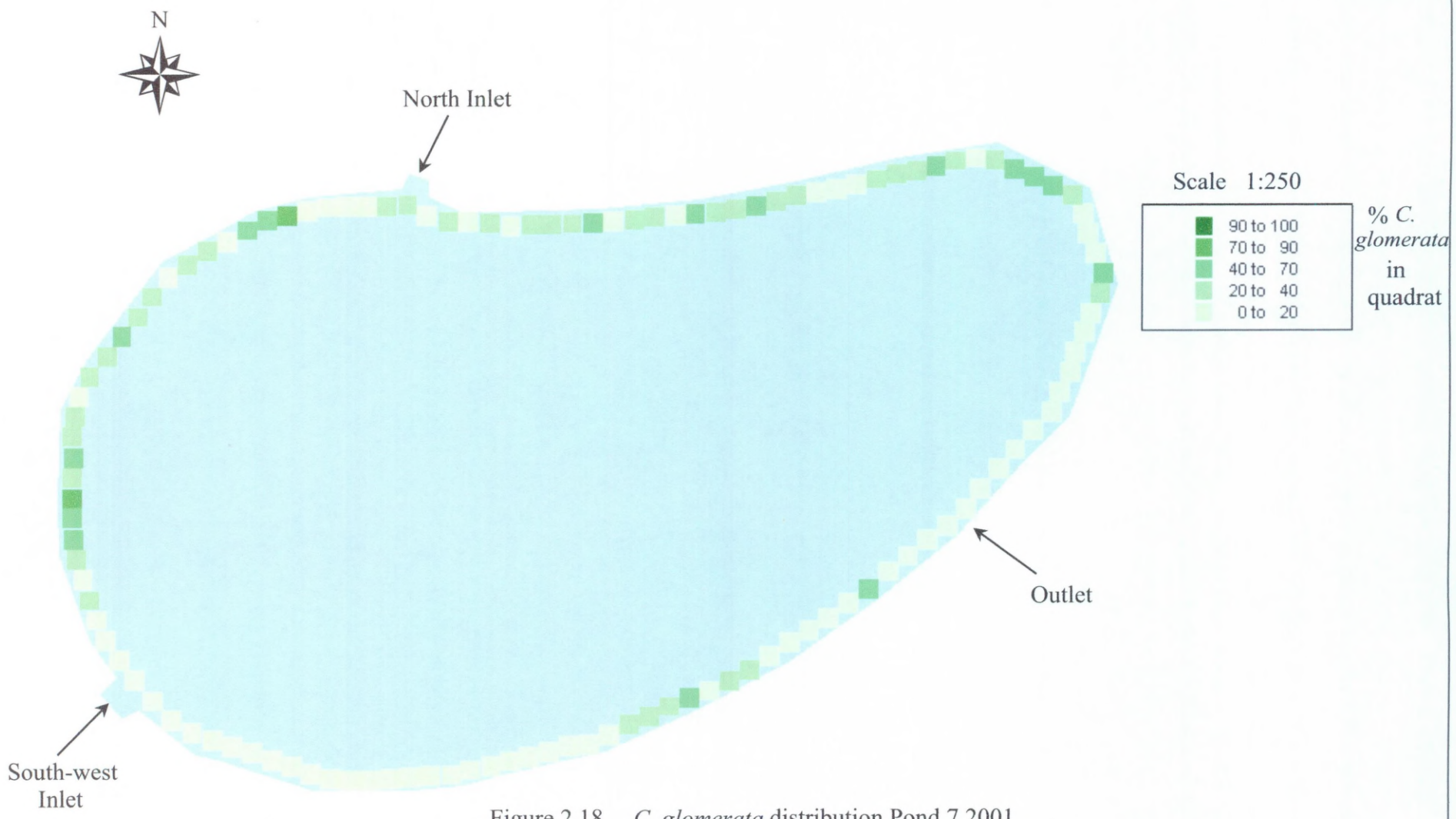


Figure 2.18 *C. glomerata* distribution Pond 7 2001

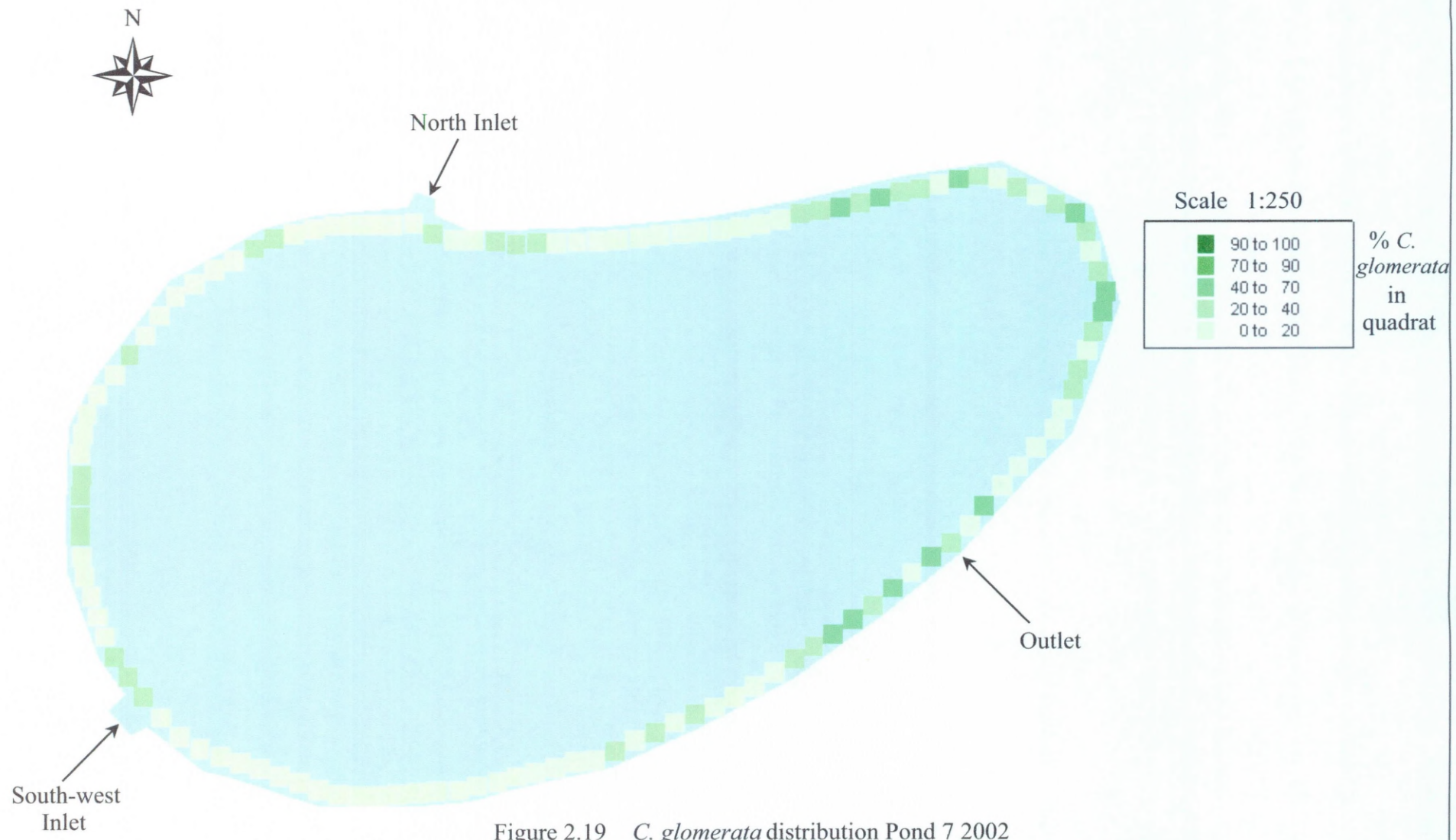


Figure 2.19 *C. glomerata* distribution Pond 7 2002

2.3.7 Pond surveys

Surveys 1A – 3C show the observational survey sheets, which were completed during each visit to the ponds throughout the spring/summer months. The surveys provide a 'snap shot' of pond parameters collected during the month of July from 2000 – 2002. The surveys take into account pollutant inputs and outputs and the overall pond conditions with respect to wildlife, litter and bank erosion. In addition, algal distribution (at pond specific areas) was assessed using the percentage of *C. glomerata* coverage within 10 quadrats (5 upstream and 5 downstream of the sampling area) and scored as a mean of 10 quadrats where trace is designated as 0 – 20% mean algal coverage (n = 10), moderate 21 – 70% mean algal coverage (n = 10) and abundant 71 – 100% mean algal coverage (n = 10). This information provides vital observational assessments of SUDS ponds throughout the sampling season, which can be obtained relatively quickly and linked with other pond data sets therefore providing an overall assessment of pond health.

Survey 1A Survey sheets for Linburn pond (July 2000) showing general observations for pond health

Weather prior to visit (3 days)	Sun / cloud
Temperature prior to visit (3 days)	16°C
Weather day of sampling	Sun
Temperature day of sampling	18°C

Inlet	RR inlet	N. east inlet	North 1 inlet	North 2 inlet	Outlet	South
Pollutants	Trace	None	None	None	None	None
Sediment	None	Trace	Trace	Trace	None	-
Discharging	Trace	None	None	None	None	-
Clogging of inlet	None	None	None	None	None	-

Sample water	Clear	Relatively clear	Turbid	Very turbid
Rip rap inlet	-	Yes	-	-
North east inlet	Yes	-	-	-
North 1 inlet	Yes	-	-	-
North 2 inlet	Yes	-	-	-
Outlet	Yes	-	-	-
South side	Yes	-	-	-

Algae	Species	Distribution (% algal coverage in quadrat; n=10)	Attached to reeds/rocks
Rip rap inlet	*C. glomerata*	Trace	Reeds
North east inlet	*C. glomerata*	Moderate	Reeds
North 1 inlet	*C. glomerata*	Trace	Reeds
North 2 inlet	*C. glomerata*	Trace	Yes to both
Outlet	*C. glomerata*	Trace	Yes to both
South side	*C. glomerata*	Abundant	Yes to both

Overall Condition	Location in pond
Litter	Yes – close to outlet attached to reeds
Vandalism	No
Erosion	Yes – south bank and close to rip rap inlet
Sediment accumulation	Trace at all inlets except rip rap inlet
Maintenance since previous visit	No evidence of maintenance
Wildlife	Mute swans, 5 signets and 4 coots
Odour	No
Children playing	No
Flies/Midges	Substantial

Abundant *C. glomerata* at south side where reed growth less dense and not up to bank. Algae mostly attached to base of *Phragmites australis* and some rocks at south side. Patches of *C. glomerata* mostly at north side close to inlets. Reed height around 6 feet and some are flowering. Visible sign of pollutants (oil film) at rip rap inlets only but only a small amount. No evidence of pollutants at outlet or south side location. Evidence of litter close to outlet trapped within the reeds.

Survey 1B Survey sheets for Linburn pond (July 2001) showing general observations for pond health

Weather prior to visit (3 days)	Dark cloud and rain with some sun
Temperature prior to visit (3 days)	15°C
Weather day of sampling	Sun and cloud
Temperature day of sampling	17°C

Inlet	RR inlet	N. east inlet	North 1 inlet	North 2 inlet	Outlet	South
Pollutants	None	None	None	None	None	None
Sediment	None	Yes	Yes	Yes	None	-
Discharging	Yes	No flow	No flow	No flow	Yes	-
Clogging of inlet	None	None	None	None	None	-

Sample water	Clear	Relatively clear	Turbid	Very turbid
Rip rap inlet	Yes	-	-	-
North east inlet	-	Yes	-	-
North 1 inlet	-	Yes	-	-
North 2 inlet	-	Yes	-	-
Outlet	Yes	-	-	-
South side	-	Yes	-	-

Algae	Species	Distribution (% algal coverage in quadrat; n=10)	Attached to reeds/stones
Rip rap inlet	*C. glomerata*	Trace	Yes to both
North east inlet	*C. glomerata*	Trace	Reeds
North 1 inlet	*C. glomerata*	Trace	Reeds
North 2 inlet	*C. glomerata*	Trace	Stones
Outlet	*C. glomerata*	Abundant	Stones
South side	*C. glomerata*	Abundant	Yes to both

Overall Condition	Location in pond
Litter	No
Vandalism	No
Erosion	Yes - South bank and close to rip rap inlet
Sediment accumulation	Yes – N. east inlet, North 1 & 2 Inlet
Maintenance since previous visit	Input straw bale winter '01
Wildlife	Pair Mute swans and 3 signets, 2 coots
Odour	No
Children playing	No
Flies/Midges	Substantial

Large patches of duckweed at north side of pond in the presence of *C. glomerata* where *Phragmites australis* growth is not as dense. Significant growth of *C. glomerata* at south side attached to both dense reeds and rocks. Reed height about 6 feet and some reeds flowering. No visible sign of pollutants at any inlets where no flow at N. east inlet, North 1 and 2 inlet. Rotting straw bale colonised with plants located close to south bank and North 2 Inlet.

Survey 1C Survey sheets for Linburn pond (July 2002) showing general observations for pond health

Weather prior to visit (3 days)	Sunshine/showers
Temperature prior to visit (3 days)	16°C
Weather day of sampling	Sunshine
Temperature day of sampling	18°C

Inlet	RR inlet	N. east inlet	North 1 inlet	North 2 inlet	Outlet	South
Pollutants	Yes	Yes	Yes	None	None	None
Sediment	None	Yes	Yes	Yes	None	-
Discharging	Yes	Yes	Yes	None	Yes	-
Clogging of inlet	None	None	None	Yes	None	-

Sample water	Clear	Relatively clear	Turbid	Very turbid
Rip rap inlet	-	-	-	Yes
North east inlet	-	-	-	Yes
North 1 inlet	-	-	-	Yes
North 2 inlet	-	-	Yes	-
Outlet	-	Yes	-	-
South side	-	Yes	-	-

Algae	Species	Distribution (% algal coverage in quadrat; n=10)	Attached to reeds/stones
Rip rap inlet	*C. glomerata*	Trace	Stones
North east inlet	*C. glomerata*	Trace	Reeds
North 1 inlet	*C. glomerata*	Trace	Reeds
North 2 inlet	*C. glomerata*	Trace	Reeds
Outlet	*C. glomerata*	Trace	Yes to both
South side	*C. glomerata*	Moderate	Yes to both

Overall Condition	Location in pond
Litter	Yes – near N. east inlet
Vandalism	No
Erosion	Yes – south bank and close to rip rap inlet
Sediment accumulation	Yes – all inlets except rip rap inlet
Maintenance since previous visit	No evidence of maintenance
Wildlife	Pair of swans and coots nesting
Odour	No
Children playing	No
Flies/Midges	Substantial

Excessive amounts of duckweed especially at south and north side. Only small patches of *C. glomerata* at south side and very little distributed throughout the rest of the pond. Lots of new *Phragmites australis* growth up to pond bank and well into pond (~ 2m). Water at all inlets very turbid due to the previous days rain. Presence of detergents at N. east inlet. Pair of swans and coots nesting in the pond. Straw bale colonised with plants located close to south bank and North 2 Inlet added spring 2002.

Survey 2A Survey sheets for Halbeath pond (July 2000) showing general observations for pond health

Weather prior to visit (3 days)	Sun/cloud
Temperature prior to visit (3 days)	16°C
Weather day of sampling	Sun
Temperature day of sampling	18°C

Inlet/Outlet Parameters	**Inlet**	**Outlet**
Pollutants	Trace	None
Sediment	Moderate	None
Discharging	Trace	None
Clogging at Inlet/Outlet	None	None

Sample water	**Clear**	**Relatively clear**	**Turbid**	**Very turbid**
Inlet (south)	-	Yes	-	-
Outlet (west)	Yes	-	-	-
North	Yes	-	-	-
East	Yes	-	-	-

Algae	**Species**	**Distribution (% algal coverage in quadrat; n=10)**	**Attached to reeds/stones**
Inlet	*C. glomerata*	Moderate	Reeds
Outlet	*C. glomerata*	Trace	Reeds
North	*C. glomerata*	Moderate	Reeds
East	*C. glomerata*	Trace	Reeds and stones

Overall Condition	**Location in pond**
Litter	Moderate – close to inlet and east side
Vandalism	No
Erosion	Yes – west bank near to inlet
Sediment accumulation	Yes - inlet
Maintenance since previous visit	No evidence of maintenance
Wildlife	Pair coots and offspring
Odour	No
Children	No
Flies/Midges	Substantial

C. glomerata on inlet concrete structure with low flow at inlet. Moderate amounts of *C. glomerata* at inlet and north side where reed growth is not so abundant. Less algae at west and east side as reed growth at times is up to the bank and quite dense. Trace amount of detergents at inlet. *Phragmites australis* growth quite dense and height of reeds reaching up to 6 feet. Moderate amount of littler trapped within the reeds at the inlet and east side of the pond.

Survey 2B Survey sheets for Halbeath pond (July 2001) showing general observations for pond health

Weather prior to visit (3 days)	Dark cloud and rain with some sun
Temperature prior to visit (3 days)	15°C
Weather day of sampling	Sun and cloud
Temperature day of sampling	17°C

Inlet/Outlet Parameters	**Inlet**	**Outlet**
Pollutants	None	None
Sediment	Yes	None
Discharging	Yes	Yes
Clogging at Inlet/Outlet	None	None

Sample water	**Clear**	**Relatively clear**	**Turbid**	**Very turbid**
Inlet (south)	-	-	Yes	-
Outlet (west)	Yes	-	-	-
North	Yes	-	-	-
East	Yes	-	-	-

Algae	**Species**	**Distribution (% algal coverage in quadrat; n=10)**	**Attached to reeds/stones**
Inlet	*C. glomerata*	Moderate	Rocks
Outlet	*C. glomerata*	Abundant	Reeds
North	*C. glomerata*	Moderate	Reeds
East	*C. glomerata*	Abundant	Reeds

Overall Condition	**Location in pond**
Litter	Yes
Vandalism	No
Erosion	Yes – close to inlet
Sediment accumulation	Yes – inlet
Maintenance since previous visit	Straw bale at west side (~ 4m out into the pond)
Wildlife	Pair swans and coots – swans nesting
Odour	No
Children	No
Flies/Midges	Substantial

Patches of duckweed at east and west side locations where *C. glomerata* is abundant. *Phragmites australis* growth quite dense and in places (west side) it is close to the pond bank. Less dense reed growth at north side location. Straw bale added to pond in winter '01 at west side anchored ~ 4m out into pond. Litter trapped among the reeds at east side. Turbid water at inlet due to rain from the previous day.

Survey 2C Survey sheets for Halbeath pond (July 2002) showing general observations for pond health

Weather prior to visit (3 days)	Sunshine/showers
Temperature prior to visit (3 days)	16°C
Weather day of sampling	Sunshine
Temperature day of sampling	18°C

Inlet/Outlet Parameters	Inlet	Outlet
Pollutants	None	None
Sediment	Yes	None
Discharging	None	Yes
Clogging at Inlet/Outlet	None	None

Sample water	**Clear**	**Relatively clear**	**Turbid**	**Very turbid**
Inlet (south)	Yes	-	-	-
Outlet (west)	Yes	-	-	-
North	Yes	-	-	-
East	Yes	-	-	-

Algae	**Species**	**Distribution (% algal coverage in quadrat; n=10)**	**Attached to reeds/stones**
Inlet	*C. glomerata*	Trace	Stones
Outlet	*C. glomerata*	Trace	Reeds
North	*C. glomerata*	Moderate	Reeds
East	*C. glomerata*	Moderate	Yes to both

Overall Condition	***Location in pond***
Litter	Yes – Inlet and east bank
Vandalism	None
Erosion	Yes – West bank close to inlet
Sediment accumulation	Yes – at inlet
Maintenance since previous visit	Presence of straw bale – west side (~ 4m into the pond)
Wildlife	Pair swans and coots (only swans nesting)
Odour	None
Children	None
Flies/Midges	Moderate

C. glomerata growth in areas where reed growth is not as dense especially at north and east side of pond. No flow at inlet with any visible signs of pollution in stagnant inlet water. Abundant growth of duckweed at west side and close to inlet with patches growing at north and east side. Bird droppings from the swans identified close to inlet up on the bank (increase of nutrients) but no algal growth in nearby area. Straw bale at west side added to pond spring 2002.

Survey 3A Survey sheets for Pond 7 (July 2000) showing general observations for pond health

Weather prior to visit (3 days)	Sun/cloud
Temperature prior to visit (3 days)	16°C
Weather day of sampling	Sun
Temperature day of sampling	18°C

Inlet	**North Inlet**	**South West Inlet**	**Outlet**
Pollutants	Trace	Trace	None
Sediment	Trace	Trace	None
Discharging	Trace	Trace	Yes
Clogging at inlet/outlet	None	None	None

Sample water	**Clear**	**Relatively clear**	**Turbid**	**Very turbid**
Inlet north	-	Yes	-	-
Inlet south west	-	Yes	-	-
Outlet east	Yes	-	-	-
South	Yes	-	-	-

Algae	**Species**	**Distribution (% algal coverage in quadrat; n=10)**	**Attached to reeds/stones**
Inlet north	*C. glomerata*	Trace	Stones
Inlet south west	*C. glomerata*	Trace	Stones
Outlet east	*C. glomerata*	Abundant	Yes to both
South	*C. glomerata*	Abundant	Yes to both

Overall Condition	**Location**
Litter	Trace
Vandalism	None
Erosion	Yes – north bank close to north inlet
Sediment accumulation	None
Maintenance since previous visit	No evidence of maintenance
Wildlife	Pair coots and offspring
Odour	None
Children	None
Flies/Midges	Trace

C. glomerata abundant at east side and south side where reed growth not so dense. High discharge flows at outlet where water is clear. Large amount of macroinvertebrates especially snails found under rocks within the pond sediments. Small amount of litter trapped within the reeds at the east side of the pond probably blown into the pond from the surrounding area.

Survey 3B Survey sheets for Pond 7 (July 2001) showing general observations for pond health

Weather prior to visit (3 days)	Dark cloud and rain with some sun
Temperature prior to visit (3 days)	15°C
Weather day of sampling	Sun and cloud
Temperature day of sampling	17°C

Inlet	**North Inlet**	**South West Inlet**	**Outlet**
Pollutants	Yes	Yes	None
Sediment	Yes	Yes	None
Discharging	Trace	Yes	None
Clogging at inlet/outlet	None	None	None

Sample water	**Clear**	**Relatively clear**	**Turbid**	**Very turbid**
Inlet north	-	-	-	Yes
Inlet south west	-	Yes	-	-
Outlet east	Yes	-	-	-
South	Yes	-	-	-

Algae	**Species**	**Distribution (% algal coverage in quadrat; n=10)**	**Attached to reeds/stones**
Inlet north	*C. glomerata*	Trace	Stones
Inlet south west	*C. glomerata*	Trace	Stones
Outlet east	*C. glomerata*	Moderate	Yes to both
South	*C. glomerata*	Moderate	Yes to both

Overall Condition	**Location**
Litter	None
Vandalism	None
Erosion	Yes – east side and close to north inlet
Sediment accumulation	Yes – both inlets
Maintenance since previous visit	No evidence of maintenance
Wildlife	Pair swans
Odour	None
Children	None
Flies/Midges	Trace

C. glomerata concentrated around the east side and south side of the pond attached to both reeds and stones. However, some algae drying out on the bank due to low levels of water in the pond – no discharge at outlet. White film on the surface of the water at north inlet and looks to be due to a cement spill at the nearby construction site. Trace amounts of *C. glomerata* at this inlet (sample taken for analysis). Sediment build up in pond close to north inlet which discharges from construction site however at the south side water is clear. Tadpoles and spawn observed close to west inlet trapped amongst the reeds. No evidence of duckweed which has colonised areas in Halbeath and Linburn pond.

Survey 3C Survey sheets for Pond 7 (July 2002) showing general observations for pond health

Weather prior to visit (3 days)	Sunshine/showers
Temperature prior to visit (3 days)	16°C
Weather day of sampling	Sunshine
Temperature day of sampling	18°C

Inlet	**North**	**South West**	**Outlet**
Pollutants	None	None	None
Sediment	Yes	Yes	None
Discharging	Yes	Yes	None
Clogging at inlet/outlet	None	None	None

Sample water	**Clear**	**Relatively clear**	**Turbid**	**Very turbid**
Inlet north	-	Yes	-	-
Inlet south west	-	Yes	-	-
Outlet east	Yes	-	-	-
South	Yes	-	-	-

Algae	**Species**	**Distribution (% algal coverage in quadrat; n=10)**	**Attached to reeds/stones**
Inlet north	*C. glomerata*	Moderate	Stones
Inlet south west	*C. glomerata*	Trace	Stones
Outlet east	*C. glomerata*	Moderate	Yes to both
South	*C. glomerata*	Trace	Yes to both

Overall Condition	**Location**
Litter	Yes – in reeds on east side
Vandalism	None
Erosion	Yes – north bank close to north inlet
Sediment accumulation	Yes at both inlets
Maintenance since previous visit	No evidence of maintenance
Wildlife	None
Odour	None
Children	None
Flies/Midges	Trace

Moderate amounts of *C. glomerata* located at north inlet and east side close to outlet attached to both reeds and stones. Low water levels in the pond so no discharge at outlet and some *C. glomerata* beached on the bank out of the water (sample taken for analysis). New growth of *P. australis* reeds at south side where growth coming up past the pond bank. Small amount of litter at east side probably blown into the pond from surrounding area. No sign of any wildlife in the pond and few macroinvertebrates visible under stones and within the pond. Straw bale added to the pond spring 2002 at north side. No evidence of duckweed which has colonised large areas in both Halbeath and Linburn pond.

2.4 Discussion

The main focus of this chapter was to develop qualitative and quantitative field based techniques, which may be used to analyse and quantify the physical, chemical and biological interactions within SUDS ponds. Data collected from this chapter may be applied to help or improve understanding of the wider functionality of SUDS pond ecosystems and biological interactions. It may lead to the development of improved pond management strategies based on biological parameters and in doing so help to enhance biodiversity within the urban aquatic landscape.

2.4.1 Chlorophyll *a*

In many aquatic systems with sufficient nutrients, there tends to be a microalgal bloom during spring months but this was not the case with the ponds studied in this project. The ponds lacked sufficient microalgal populations and chlorophyll *a* levels in water samples were at the limits of assay detection throughout the three-year period (2.3.1). The low levels of microalgae detected in the ponds during the three-year study period, as depicted by chlorophyll *a* analysis may be due to SS inputs mainly from construction sites and soil erosion (Linburn pond and Pond 7) (Figure 2.6), as discussed by Maitland (1990). Furthermore, short pond water retention times and insufficient residual phytoplankton populations from the previous year can also influence algal blooms (Talling, 1999; Reynolds & Irish, 2000). Suspended solids affect primary production in freshwaters by reducing light penetration where photosynthesis is only possible in the upper layers as silt settles on the pond bottom blanketing out the microalgae. Very high levels of suspended solids are usually episodic and are related to high rainfall and floods within the catchment. However, the water normally clears after such an episode and much of the material that has settled on the bottom gradually washes away or is

incorporated into the normal substrate (Maitland, 1990). In addition to SS inputs, dry summers with low flows will fail to carry significant phytoplankton inocula to the ponds and very wet autumn/winters dilute pond inocula. Both cases will cause a decrease in pond microalgal content for the next year (Reynolds & Irish, 2000). Furthermore, the ponds in this study were designed to have a short water retention time of 2 – 3 weeks, which can lead to the episodic wash out of microalgae and SS inputs especially during high rainfall events (Talling, 1999). It is likely that a combination of these events has occurred in the ponds resulting in insufficient microalgal populations despite an adequate supply of nutrients for algal growth (Figure 2.7 – 2.9).

2.4.2 Chlorophyll *a/b*

Dense growth of *C. glomerata* is common in eutrophic freshwaters especially in summer (Cate *et al.*, 1991) and this was also the case for the ponds studied despite Halbeath pond falling into the oligotrophic - mesotrophic category (2.3.3). Due to the structure of *C. glomerata* oxygen bubbles can become trapped amongst the filaments causing the mats to float on the surface. However, this growth habit exposes the alga to high irradiance and causes the alga to turn yellow in colour due to a reduction in chlorophyll levels (Sze, 1998) by photooxidation. This event was observed with *C. glomerata* filaments in the ponds and may help to explain the decrease in chlorophyll *a/b* ratios from the normal chl *a/b* ratio of 2.6 (Larkum & Barrett, 1983) for a number of algal samples (Table 2.1). Exposure to prolonged high irradiance will inhibit photosynthesis and damages the photosynthetic system (Dodds & Gudder, 1992). To reduce such adverse effects, some carotenoids function in photoprotection by screening the chloroplasts and deactivating highly reactive forms of oxygen ($^1O_2$) before cellular damage occurs (Sze, 1998) (SOD also plays an important role see 4.1.1). However, it is

unlikely that *C. glomerata* in the ponds suffered from photoinhibition due to the alga undergoing self-shading (Wiencke & Davenport, 1987). As the thallus size of the alga increases, self-shading can occur resulting in an uneven light field where filaments other than those at the very top of the mat may experience photoinhibition. *C. glomerata* samples displaying increased chl *a/b* ratios of 3.3 (June 2001 – South) and 2.9 (September 2001 – West) (Table 2.1) indicate that they were exposed to low irradiance levels due to shading from the aquatic plant *Phragmites australis*.

2.4.3 Water quality

Aquatic environments that have a pH > 8.5 are normally associated with eutrophication and hence may contain elevated phosphate levels (Chapman, 1996). From the data, the majority of Linburn pond and Pond 7 samples with pH > 8.5 have correspondingly higher orthophosphate levels (> 0.5 $mg.l^{-1}$) and are categorised as eutrophic - hypertrophic, as compared to Halbeath pond that has a pH < 8.5, coupled with lower phosphate levels (< 0.5 $mg.l^{-1}$) and classed as mesotrophic (Figures 2.4, 2.9 and Table 2.1). In addition, photosynthesis during moderate light intensity and respiration during low light can cause the pH to fluctuate daily (Lewis & Wang, 1997) especially during the summer months when algal abundance is high. Unfortunately, the daily pH fluctuations for each pond were not recorded (due to the travelling distance to the pond sites), but from the data in Figure 2.4, the pH does fluctuate to some extent between months, however, it cannot be assumed that this is due to photosynthesis/respiration of algae especially during the summer months. Generally, pond pH is within the range pH 7 – 8.5 providing ideal conditions for abundant growth of algae (Kozitskaya & Komarenko, 1995) including *C. glomerata* which favours growth in pH > 7.0 (Pitcairn & Hawkes, 1973; Dodds, 1991).

The majority of water samples collected from the ponds either fell between 500 to 1000 $\mu S.cm^{-1}$, or exceed this value (Figure 2.5). Generally, the conductivity of most freshwaters can range from 10 to 1,000 $\mu S.cm^{-1}$ where values in excess of 1,000 $\mu S.cm^{-1}$ are especially common in polluted waters, or those receiving large quantities of land runoff (Gray, 1999; Chapman, 1996). It is thought that samples in excess of 1000 $\mu S.cm^{-1}$ are likely to be as result of salt applications on road surfaces (as indicated on Figures 2.5 & 2.10) especially during the winter and early spring months.

Samples that display high suspended solid concentrations of > 100 $mg.l^{-1}$ (Figure 2.6) are likely to be associated with construction site solid inputs and soil erosion (Gray, 1999; Novotny, 1998). Increases in suspended solids can reduce light penetration depressing photosynthesis and thus reducing primary production (Gray, 1999). This consequence can have serious implications for food web interactions within aquatic systems (Chapter 6). Generally, suspended solid concentrations from the ponds were < 50 $mg.l^{-1}$ (Figure 2.6), at these concentrations they are unlikely to cause adverse effects to fish health (Alabaster, 1980). In addition, suspended solid concentrations were lower at the outlet than at the inlets (for all ponds,) indicating efficient pond sedimentation (Shatwell & Cordery, 1999). However, efficient sedimentation can pose a problem in ponds with respect to eutrophication due to the re-suspension of phosphorous back into the water column especially during high flows (Lewis & Wang, 1997; Jenson & Anderson, 1992) and can also result in the accumulation of heavy metals over a prolonged period of time (Novotny, 1998).

Ammoniacal nitrogen assessment of surface waters provides a useful indicator of organic pollution as it is normally found in very low concentrations (< 0.1 $mg.l^{-1}$) in unpolluted waters. Surface water concentrations in excess of > 3 $mg.l^{-1}$ generally

indicate organic pollution from domestic sewage (foul to storm cross-connections) and fertiliser runoff (Chapman, 1996). Both Halbeath pond and Pond 7 fall within the normal ammoniacal nitrogen range for surface waters (2-3 $mg.l^{-1}$) but Linburn pond suffered from organic pollutant inputs with ammoniacal nitrogen concentrations > 10 $mg.l^{-1}$ (Figure 2.7). Research carried out by Cate & Schreurs (1991) and Robinson & Hawkes (1986) has reported that *C. glomerata* is sensitive to high ammoniacal nitrogen levels, which may prove toxic to the species. However, it is unlikely that the levels detected in the ponds were detrimental to the growth and survival of *C. glomerata.* High ammoniacal nitrogen levels can cause problems with dissolved oxygen levels in the ponds (Davis *et al.*, 1991; Sawyer *et al.*, 1994). Oxidation of ammoniacal nitrogen to nitrite and nitrate requires oxygen where a severe reduction in oxygen levels can compromise the biological stability of the ponds.

Generally, TON concentrations (include both NO_3^- and NO_2^-) from Figure 2.8 show a similar pattern to that of ammoniacal nitrogen (NH_3-N) (Figure 2.7). In addition to organic pollutant inputs from human waste (foul to storm cross-connections) and fertiliser runoff, TON is recycled within the ponds as a result of algal/plant decomposition and resuspension from the sediments. Decomposition of plants/algae release organic nitrogen, which is converted to ammoniacal nitrogen and then oxidised to nitrate (available form for plants and algae) (Figure 1.10) (Davis & Cornwell, 1991). Therefore, enhanced concentrations of ammoniacal nitrogen results in increased TON levels and with the addition of nitrogen inputs from external sources (organic pollution) can significantly impact the nitrogen cycle causing excessive plant and algal growth. TON concentrations in excess of 0.2 $mg.l^{-1}$ are known to stimulate algal growth (Chapman, 1996). Figure 2.8 show that the ponds contain sufficient nitrogen for

abundant algal growth with concentrations > 0.2 $mg.l^{-1}$ indicating that they may be susceptible to organic pollutant inputs.

Figure 2.9 demonstrates a second peak occurring in orthophosphate (inorganic phosphorous) concentration for all graphs during the winter months, probably as a result of minimal algal activity, algal death and reed die back, which release orthophosphate back into the water column and sediments (Talling, 1999). The data also indicates a depletion in orthophosphate levels during the summer months when algal growth reaches maximal levels; this was anticipated as it is a pre-requisite for algal growth (Talling, 1999). However, it must be noted that there is an increase in orthophosphate levels during one month for each pond (Linburn pond – March 2001; Halbeath pond and Pond 7 – July 2001) and is most likely to be due to phosphorus remobilisation from the sediments (Shatwell & Cordery, 1999) (as indicated on Figure 2.9). During the summer when plant and reed growth (*Phragmites australis*) is maximal, soluble phosphorus is removed from the water column by the plants causing sediment-bound orthophosphate to be released (Lewis & Wang, 1997) in its available form (inorganic) for algal growth. Furthermore, a handful of samples from Linburn pond display high phosphate concentrations exceeding 2 $mg.l^{-1}$ (due to phosphorous remobilisation from the sediments, algal death and reed dieback), which at these concentrations are toxic to *C. glomerata* (Robinson & Hawkes, 1986). However, it is unlikely that these concentrations had a detrimental effect on the growth of *C. glomerata* as orthophosphate levels generally fell below 2 $mg.l^{-1}$ for most Linburn pond samples.

Similar concentration patterns for both chloride and conductivity parameters were observed (Figures 2.10 & 2.5) indicating that seasonal fluctuations in chloride levels are likely to be due to road salting during winter and early spring months (Figure 2.10).

Pristine freshwater chloride concentrations are usually < 10 $mg.l^{-1}$ where higher concentrations such as those detected from the ponds can occur near sewage and waste outlets (Chapman, 1996).

2.4.4 Pond sediment surveys

Pond sediment data collected on a yearly basis (1999 – 2001) by Dr Kate Heal and shared for the purpose of this project, indicates that certain metals will accumulate in the ponds as they age (Appendix 4B - 4D) (Heal, 2002). All of the pond sediments analysed during 2001 decreased in both nitrogen and phosphorous concentration (Appendix 4D). It is likely that the decrease in sediment nitrogen and phosphorus concentrations was due to plant uptake by the abundant and rapidly growing common reed, *Phragmites australis*. Furthermore, the relative decrease in abundance of *C. glomerata* (Figures 2.11 – 2.19) may be due to the accumulation of heavy metals in the sediments. Whitton (1979) identified that *C. glomerata,* although tolerant of organic and nutrient pollution is the most sensitive of all algae tested to heavy metal pollution (Cu, Zn, Pb). This observation concurs with quadrat data that shows a decrease in algal abundance from 2000 – 2002 (Figures 2.11 – 2.19) in addition to the ponds containing increased concentrations of copper, zinc or lead (Appendix 4B - 4D).

2.4.5 Critique of pond surveys and algal distribution

The incorporation of qualitative surveys (designed by the SUDS consortium) for the ponds proved to be an important source of data for this project, as it highlights key components of the ponds, which may have been overlooked by other sampling regimes (e.g. poor management). The most valuable observations from the survey sheets for this project are notes on overall pond condition and general comments relating to plant

growth/accumulation and human inputs and management regimes, which can potentially interfere with ecosystem function. The survey sheets could, however, be improved biologically, by adding sections relating to plant growth and human/management impacts. Obtaining sufficient information in these areas could supplement and link with the physical and chemical data previously gathered from the ponds, to help recommend management strategies for the ponds. However, a major drawback to the use of these surveys in monitoring programmes is that they are very subjective (based on observer opinion) and the components are difficult to quantify. But, these can be overcome if they are used in conjunction with other data sets including water quality, algal distribution, and hydrology and sediment data. These surveys can be viewed as an invaluable source of information for aquatic monitoring programmes and in addition, they can highlight areas for improvement and help to recommend strategies for adequate pond management.

Duckweed (*Lemna, Spirodela, Wolffia spp.*), small floating plants that can form a solid mat preventing light penetration for algal growths, (Hancock & Buddhavarapu, 1993; Campbell & Ogden, 1999) was not observed in the ponds during year 2000 (Surveys 1A - 3C). However, the presence of this plant increased in Linburn and Halbeath pond during year 2001 and 2002 (Surveys 1A – 2C). During sampling, it was observed that this plant colonised areas where algal growth was prevalent the previous year, usually in areas where the growth of *Phragmites australis* was not dense. Competition for light and nutrients (Campbell & Ogden, 1999) may have occurred between duckweed and *C. glomerata* and due to duckweed's susceptibility to wind/wave movements (Campbell & Ogden, 1999) it is likely that the plant colonised areas with less dense *Phragmites australis* thus maximising its growth and subsequently reducing light and nutrients for *C. glomerata*. In addition, algal growth may have been reduced in the ponds due to an

increase in growth and density of the common reed *Phragmites australis*. Initially, *Phragmites australis* was introduced into the ponds for aesthetic purposes and to act as a barrier to children playing (Pond Action, 2000). However, it was observed that as the ponds became older, the growth and density of the reeds became greater, further reducing light penetration and nutrients for algal growth especially at the pond fringes (Surveys 1A - 3C). In addition, straw bales (barley) were added to the ponds during the sampling period in an attempt to control algal growth (Surveys 1A - 3C). However, this action was not properly managed, as bales were added at times when algal biomass was minimal (winter months – Halbeath and Linburn pond 2001), and were left unattended for long periods, therefore, it is unlikely that the addition of bales had an effect at preventing algal growth. However, it is difficult to assess whether the decrease in algal abundance was due to the release of polyphenolic compounds from decomposing straw inhibiting algal growth (Pillinger *et al.*, 1994), or a combination of other factors as discussed previously.

Assessing the distribution of *C. glomerata* at the pond fringes using quadrat analyses proved to be a successful technique for this project, as the data not only highlights 'hot spot' areas of algal growth/absence in relation to external factors (i.e. sediment and water quality, plant growth etc.), but it also shows the subtle changes that occur at the algal level in the ponds over a three year period. These were mainly attributed to pond environmental change. These changes may have been overlooked if the quadrat analyses were not performed and also highlights the importance for development and modification of techniques, which have been successfully used in other fields of research.

The data collected shows an overall decline in *C. glomerata* abundance in the period from 2000 – 2002, despite there being sufficient nutrients for algal growth (Figures 2.7 – 2.9). This decline may be due to a number of factors as indicated previously, including the accumulation of heavy metals in the sediments, for which this alga is sensitive, the abundance of duckweed (*Lemna, Spirodela, Wolffia spp.*), and *Phragmites australis* competing for light, space and nutrients, ill-advised management strategies and human impacts such as the removal of *C. glomerata* from the ponds, the use of straw bales to control algal growth and fish inputs. All, or a combination of these factors could account for the steady decline of *C. glomerata* in the ponds. The interference of humans and management has an important implication for maintaining natural food web interactions and ecosystem balance. As previously mentioned (1.3), algae including *C. glomerata* are important primary producers in an aquatic system, providing food and habitats for macroinvertebrates. The physical removal of *C. glomerata* from the ponds and the introduction of 60 carp in Linburn pond during 2001 by a local resident, can lead to a perturbed pond ecosystem, whereby a food shortage of both algae and zooplankton can deplete macroinvertebrates and bird species and subsequently effect pond biodiversity (Figure 6.2). The removal of algae within the ponds can also provide an opportunity for excessive duckweed, *Phragmites australis* and non-native plant species accidentally introduced in the soil of other plants to grow (Pond Action, 2000). This can reduce light penetration, cause fluctuations in oxygen levels, and disrupt the pond ecosystem balance and cause damage to natural vegetation communities. The issue of ill-advised management/planting regimes, education/communication of SUDS with local residents and recommendations for pond management with a view to improving SUDS sustainability in a biological context will be discussed in greater detail in the general discussion (Chapter 6).

2.5 Conclusions

The focus of this chapter was to develop qualitative and quantitative techniques to help analyse and elucidate biological interactions within SUDS ponds, leading to the improvement of pond management strategies. This was to be achieved through the assessment of water quality, monitoring algal chlorophyll composition and distribution within the ponds, general pond observations and site visits.

Data from the ponds indicate that planktonic microalgal populations were extremely low despite the ponds containing adequate nutrients. It is hypothesised that this may be due to suspended solid inputs, insufficient residual (inocula) phytoplankton populations from the previous year and the short pond water retention times. Furthermore, chlorophyll *a/b* ratios for a number of *C. glomerata* samples fell below the normal chlorophyll ratio of 2.6:1 therefore indicating excessive exposure to high irradiance.

Water quality data from the ponds show a pH level in the range of pH 7 – 8.5, with conductivity and chloride levels within the ponds being influenced by road salting inputs during the winter/early spring months. Relatively high suspended solids were detected sporadically in each pond during and after heavy rainfall. These were likely to be due to construction site runoff and soil erosion; however, the level of suspended solids was not sufficiently high to cause adverse effects to fish health (Alabaster, 1980). Overall, the ponds suffer from organic pollutant inputs (fertiliser runoff, sewage and algal/plant decomposition) and therefore show fluctuations in both ammoniacal nitrogen and total oxidised nitrogen levels. Orthophosphate levels also fluctuate between months due to of algal/plant uptake, algal/plant death and resuspension from the sediments during high flows and heavy rainfall events.

Sediment and quadrat data demonstrated a yearly accumulation of heavy metals in the sediments with a subsequent reduction in algal abundance. The decline in algal growth may be due to heavy metal build up in the sediments; excessive plant growth (duckweed and *Phragmites australis*) competing for light, space and nutrients and the addition of straw bales to control algal growth.

The incorporation of qualitative observational surveys for the ponds proved to be an important source of data for this project, as they highlighted key components of the ponds such as plant growth, which may have been overlooked by other sampling regimes. These surveys can be used in combination with the physical and chemical data obtained in this project to improve the understanding of pond dynamics and to recommend management strategies for the ponds thus helping to enrich pond diversity and improve biological sustainability within the urban landscape. However their design may be improved through the use of more objective questioning and recording criteria.

Chapter 2 highlighted a wide range of integrated and confounding issues that will be explored in a biological context throughout the thesis. The holistic concept of improved SUDS sustainability through revised management techniques that take into account biological components will be discussed in more detail in Chapter 6.

Chapter 3 DEVELOPMENT OF A TOTAL ANTIOXIDANT ASSAY FOR MONITORING *C. GLOMERATA* RESPONSES IN URBAN PONDS

3 Introduction

The development of a simple, reliable and robust total antioxidant assay is a novel tool that can be used to assess the total antioxidant profile of *C. glomerata* exposed to stress. The assay can be utilised in an antioxidant programme, to initially and rapidly screen total antioxidant status in a large number of samples (Miller *et al.*, 1996; Re *et al.*, 1999). The method has the advantage of identifying samples of interest simply and quickly, reducing time such that subsequent and more detailed antioxidant analyses can be performed on particular samples. This is the first time that a total antioxidant assay has been applied to algae and a major component of this chapter was the development of an extraction technique for *C. glomerata* and the validation of this assay for algae.

In the context of this study, it is hoped that the development of the total antioxidant assay will be able to provide an insight into specific "hot spot" environmental impact locations in ponds (exposed to pollutant inputs) within particular months (of the algal growth seasons) using *C. glomerata* as the test species. In addition, the performance and applicability of the total antioxidant assay, as a tool to investigate *C. glomerata* stress responses in urban ponds will be assessed with a view to using algae in phytoremediation programmes.

3.1 Assessment of antioxidant assays

Several assays have been developed for measuring total antioxidant activity of various body fluids, extracts and compounds (Whitehead *et al.*, 1992; Wang *et al.*, 1996; Rice-Evans & Miller, 1994). Some methods involve the generation of a radical, produced through a variety of mechanisms and the measurement of a range of end points of oxidation/reduction reactions at a fixed time point or over a time series (Re *et al.*, 1999; Rice-Evans & Miller, 1994).

Generally two types of approach have been taken; either the inhibition assay where radicals are generated in the presence of an antioxidant system and the extent of inhibition of radical formation by the antioxidant is measured at a fixed time point (Miller & Rice-Evans, 1997; Miller *et al.*, 1993), or a scavenging assay. Thus, the radical accumulates in the absence of an antioxidant measured spectroscopically to a fixed absorbance point and the extent of radical scavenging with addition of an antioxidant is measured over a time series (Re *et al.*, 1999; Miller *et al.*, 1996).

3.1.1 Inhibition assay

The principle of the inhibition assay is based on the formation of a ferrylmyoglobin radical generated from metmyoglobin and hydrogen peroxide. This in turn, converts the compound 2,2' –azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) ABTS to produce a radical cation $ABTS^{\cdot+}$. The generation of $ABTS^{\cdot+}$ is measured by an increase in absorbance at 734nm therefore the antioxidant-induced inhibition of absorbance is directly related to the antioxidant capacity of the extract being tested and compared to a blank at a fixed time point (10min) (Strube *et al.*, 1997). However, this assay has been criticized on the basis that faster reacting antioxidants might contribute to the reduction

of the ferrylmyoglobin radical before it has the opportunity to convert ABTS to $ABTS^{\bullet+}$ radical cation. Therefore a new and improved assay based on generating the ABTS radical prior to the addition of antioxidants in the reaction (scavenging assay) was developed by Re *et al.* (1999), and applied in the present study (3.1.2).

3.1.2 Scavenging assay

In the case of this study the scavenging assay is based on a decolourisation assay whereby the ABTS radical is generated directly in a stable form prior to reaction with antioxidants. Direct production of the blue/green $ABTS^{\bullet+}$ chromophore is generated through the reaction between ABTS and potassium persulfate. Antioxidant-induced scavenging of the pre-formed ABTS radical reduces $ABTS^{\bullet+}$ to ABTS coupled with a decrease in absorbance at 734nm. The extent of reduction is dependent on antioxidant activity, concentration of the antioxidant and duration of the time series reaction (Re *et al.*, 1999).

3.1.3 Development of algal extraction techniques

Extracting intracellular material from macroalgae and more specifically *C. glomerata* for use in bioassays can prove problematic due to their robust structure. The development of an efficient, reliable and reproducible technique for the extraction of antioxidant enzymes from *C. glomerata* is essential for determining total antioxidant (3.3.2.1 – 3.3.2.3) and specific antioxidant enzyme status (4.3) in *C. glomerata* exposed to biotic and abiotic stress. The technique first applied to *C. glomerata* was the extraction methodology originally developed by Benson & Roubelakis-Angelakis (1992; 1994). However, removing intracellular material from *C. glomerata* requires extra physical disruption (3.2.2.2 – 3.2.2.4) and therefore the techniques used in this

project (3.2.2.5) can be considered an adaptation of the original method (Benson & Roubelakis-Angelakis, 1992; 1994).

3.2 Materials and Methods

3.2.1 *C. glomerata* collection

C. glomerata samples were collected with forceps from strategic areas (i.e. susceptible to pollution) within the pond and inlets and outlets during maximal algal growth periods (spring-summer). Samples were collected from Linburn pond sampling locations during the months of June, July and September 2000. Pond 7 samples were collected from the designated locations during the months of July and August 2000. Halbeath pond was sampled during 2000 and 2001 from the same locations during June, July, August and September. Algal samples were not collected from Linburn pond or Pond 7 during August 2000, June and September 2000 respectively, as there was insufficient algal biomass. *C. glomerata* samples were collected from Halbeath pond only during summer 2001, due to very low algal biomass at Linburn pond and Pond 7.

3.2.1.1 *C. glomerata* processing

To prevent sample dehydration, the algae were placed in polythene bags containing a small amount of pond water, and returned to the laboratory immediately. Within 24 hours of collection, the algal samples were initially washed in tap water, viewed under the microscope (Leitz) to confirm the identification of *C. glomerata*. They were then re-washed in deionised water to remove debris and any trapped macroinvertebrates. Once the samples were blotted dry, they were weighed into 1g aliquots to enable analyses on a fresh weight basis. For long-term storage (3-9 months) the aliquots were

deep-frozen at –85°C until required, for short-term storage (1-3 months) aliquots were frozen at –20°C until required.

3.2.2 Extraction techniques

The techniques used in this project for the extraction of intracellular material from *C. glomerata* are modified versions (3.2.2.2 – 3.2.2.5) of the original method developed by Murphy and Huerta (1990) for cultured rose cells. At all times throughout the extraction procedures, care was taken to minimise thawed elapsed time by ensuring that all algal samples were contained on ice until required and then immediately returned to the –20°C freezer after use.

3.2.2.1 Extraction buffer

Algal samples during all extraction procedures (3.2.2.2-3.2.2.5), were treated with varying volumes of chilled (on ice) potassium phosphate (50mM) extraction buffer (pH7) prepared with KH_2PO_4 (potassium di-hydrogen orthophosphate) and K_2HPO_4 (di-potassium hydrogen orthophosphate) supplemented with 1mM $CaCl_2$, 1mM KCl and 1mM EDTA adjusted to pH7 (Murphy & Huerta, 1990).

3.2.2.2 Mechanical extraction

Algal aliquots, once thawed on ice at room temperature, were transferred to a ceramic mortar and pestle (on ice) in the presence 4ml of extraction buffer (3.2.2.1). Filaments were ground for pre-selected periods of 1min, 5min and 1 min. Following grinding the algal filaments were viewed under a compound microscope to assess the degree of filament/membrane disruption.

3.2.2.3 Homogenisation and liquid nitrogen

Prior to grinding, algal filaments were placed in a mini-blender to help shear the filaments and cause membrane damage. The filaments were then transferred to a mortar and pestle (on ice) containing 4ml of extraction buffer (3.2.2.1). Liquid nitrogen (LN) was added to snap freeze the sample and further aid in the grinding process. Grinding continued for time periods of 1min, 5min or 10min periods where the degree of filament/membrane disruption following grinding was assessed using microscopy.

3.2.2.4 Sonication

Algal filaments were first ground with LN in a mortar and pestle containing 4ml of extraction buffer (3.2.2.1) the filament suspension was then sonicated (on ice) to rupture the filament membranes. Throughout the sonication procedure care was taken to record any temperature rises within the sample. The suspension was sonicated with a 4mm microtip probe at pre-selected frequencies not exceeding the microtip limit of 40% during time periods of 15sec to 3min with an on/off pulse of 5sec to reduce temperature elevation within the sample. Following sonication, the suspension was viewed under a microscope to assess the degree of membrane/filament disruption.

3.2.2.5 Liquid nitrogen and sonication

The algal filaments were cut with scissors into approximately 5mm lengths and added to a mortar and pestle (on ice). Whilst grinding, four sequential applications of LN with 8ml of extraction buffer (3.2.2.1) were added to aid filament/membrane disruption. Following grinding, the suspension was sonicated (on ice) (600 Cole Parmer 4mm microtip) at 30% amplitude with pulse on/off for 5sec for a total sonication period of

2min. To remove large algal aggregates, the extract was filtered through a Grade 1 Whatman filter with the aid of a vacuum pump (Neuberger-N86 KN18) and centrifuged in a cold room (5°C) using a pre-cooled micro-centrifuge (5°C) (Jouan A14) at 14,000rpm for 10min. The supernatant was transferred to a fresh Eppendorf (2 ml) and stored at –20°C until required. To recover algal extracts for assaying, extracts were removed from the –20°C freezer and thawed over ice. Material was maintained on ice and once the appropriate volume of extract was removed for assaying the unused portion of extract was immediately returned to the –20°C freezer.

3.2.3 Soluble protein determination

Total soluble protein was evaluated using the Coomassie blue dye method (Bradford, 1976). 100mg Coomassie Brilliant Blue G-250 was dissolved in 50ml ethanol (95% v/v) and 100ml phosphoric acid (85% w/v). The total volume of the reagent was made up to 1 litre with dH_20, decanted into a foil wrapped bottle and used immediately in the protein assay. Protein standards in the range of 25-2000$\mu g.ml^{-1}$ protein were prepared from ampoules containing 2$mg.ml^{-1}$ bovine serum albumin (BSA) (Sigma-Aldrich). For all assays, a 0.05ml sample/standard was added to 2.5ml of reagent, vortexed and read in a spectrophotometer (Cecil Series 2 CE292) at an absorbance of 595nm. Assays were performed in triplicate and standardised to 1ml.

3.2.4 Total antioxidant assay

The total antioxidant assay is based on the generation of an $ABTS^{\cdot+}$ [2,2'-azino(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation where the addition of antioxidants to the radical reduces it to ABTS (Re *et al.*, 1999). The assay compares the performance of a standard (Trolox - a vitamin E analogue that is water soluble) to algal

extracts in their ability to scavenge $ABTS^{\cdot+}$. The total antioxidant activity of the algal extracts was determined in the supernatant after extraction with liquid nitrogen and sonication (3.2.2.5). ABTS was dissolved in dH_2O to a 7mM concentration. The ABTS radical cation was produced by reacting the ABTS stock solution with 2.45mM potassium persulfate (final concentration) and incubating the solution in the dark at room temperature for 12-16hrs before use. The radical stock solution was diluted with a 5mM solution of phosphate buffered saline (PBS) (pH 7.4) to obtain a spectrophotometric absorbance value of 0.700 ($\pm$ 0.020) at 734nm. For all assays, a 10µl sample/standard was added to 1ml $ABTS^{\cdot+}$ solution ($A_{734}nm = 0.700 \pm 0.020$) and an absorbance reading taken exactly 1min after initial mixing until the decrease in absorbance ceased. Assays were performed in triplicate and validation runs were performed against PBS. Trolox standards (Calibochem) were prepared in PBS to cover the range of 1-15µM.10mls^{-1} PBS. Total antioxidant activity was expressed as the rate of decrease in absorbance/min.mg protein and as the mean of three replicate samples.

3.2.5 Data analysis using Minitab version 13

Results were analysed separately for each pond assuming a general linear model and two way analysis of variance (ANOVA) using month and location as factors with and without interaction as appropriate (Zar, 1996). ANOVA assumes data within each level are normally distributed with the same standard deviation. The significance level was assumed to be 5% and P values are reported in ranges such as $p < 0.001$; $p < 0.01$; $p < 0.05$ and $p > 0.05$ (Elliott, 1977). Pairwise comparisons within each pond were investigated using Tukey's method (Zar, 1996) with a 5% overall error rate. All response variables for Linburn pond and Pond 7 satisfied both of these assumptions allowing comparisons to be made between sampling month and sampling location with

respect to protein levels and total antioxidant activity. Halbeath data did not satisfy ANOVA assumptions, therefore data were power transformed to satisfy both normal data distribution and equal standard deviation. All calculations were analysed using Minitab v.13 (Minitab, USA).

3.3 Results

3.3.1 Development of extraction techniques

Examination under a light microscope demonstrated that grinding algal samples on ice was ineffective for the complete rupture of a large proportion of the filaments. However, there was some success, therefore grinding of the sample was employed in combination with other mechanical techniques. Blending prior to grinding proved to be ineffective as the algal strands wrapped round the blender blades resulting in loss of algal biomass. However, the application of liquid nitrogen proved effective, as the samples were maintained in an ultra-cold state during grinding reducing the likelihood of enzyme denaturation due to temperature elevation, aiding filament disruption. However, upon visual inspection, the strands were not completely shattered. Applying a freeze thaw cycle with liquid nitrogen in combination with grinding ensured that the sample maintained an ultra-low temperature, but a small proportion of filaments still remained intact. In contrast, applying liquid nitrogen in combination with grinding and sonication (on ice) provided a reliable and robust protocol for the complete extraction of algal intracellular material. Ultra – low temperatures were achieved whilst grinding due to liquid nitrogen addition, and pulsing on ice (0°C) during sonication ensured minimum temperatures were maintained. This protocol was employed throughout the study as the standard method for extracting intracellular material from *C. glomerata*.

3.3.2 Protein and total antioxidant activity analysis

The results have been presented on a pond-by-pond basis to facilitate interpretation of differences in protein levels and total antioxidant activity between each pond. This allows variations between sampling months, sampling location and in the case of Halbeath year-to-year comparisons to be investigated. Key parameters assessed within the ponds were variables related to: (a) differences between sampling times during the algal growth season and (b) differences between pond locations and (c) year to year comparisons for Halbeath pond.

3.3.2.1 Linburn Pond

Significant differences were detected between mean protein levels and sampling month ($F_{2,43} = 3.27$; $p < 0.05$) but not significant for pond sampling location ($F_{2,48} = 2.94$; $p > 0.05$) when data were analysed using a general linear model of a two way ANOVA and (Figure 3.1 A). However, when monthly protein data were compared using one-way ANOVA, no significant differences were detected between sampling month and protein levels ($F_{2,48} = 2.94$; $p > 0.05$).

Significant differences were detected between the mean total antioxidant activity, sampling month and pond sampling location when data were analysed using a general linear model of a two way ANOVA with Tukey's pairwise comparisons. Assessment of sample months with Tukey's analyses identified significantly lower total antioxidant activity from samples collected during September 2000 than samples collected during June and July 2000 ($F_{2,43} = 4.91$; $p < 0.05$). Comparison of sample location data with Tukey's analyses identified an increase in antioxidant activity from outlet and south

side locations and significantly lower total antioxidant activity in rip rap inlet samples as compared to other sampling locations ($F_{5,43} = 4.78$; $p < 0.001$). (Figure 3.1 B).

No significant Pearson correlations were detected between protein levels and total antioxidant activity in *C. glomerata* samples collected from Linburn pond ($r = -0.0072$; $p > 0.05$).

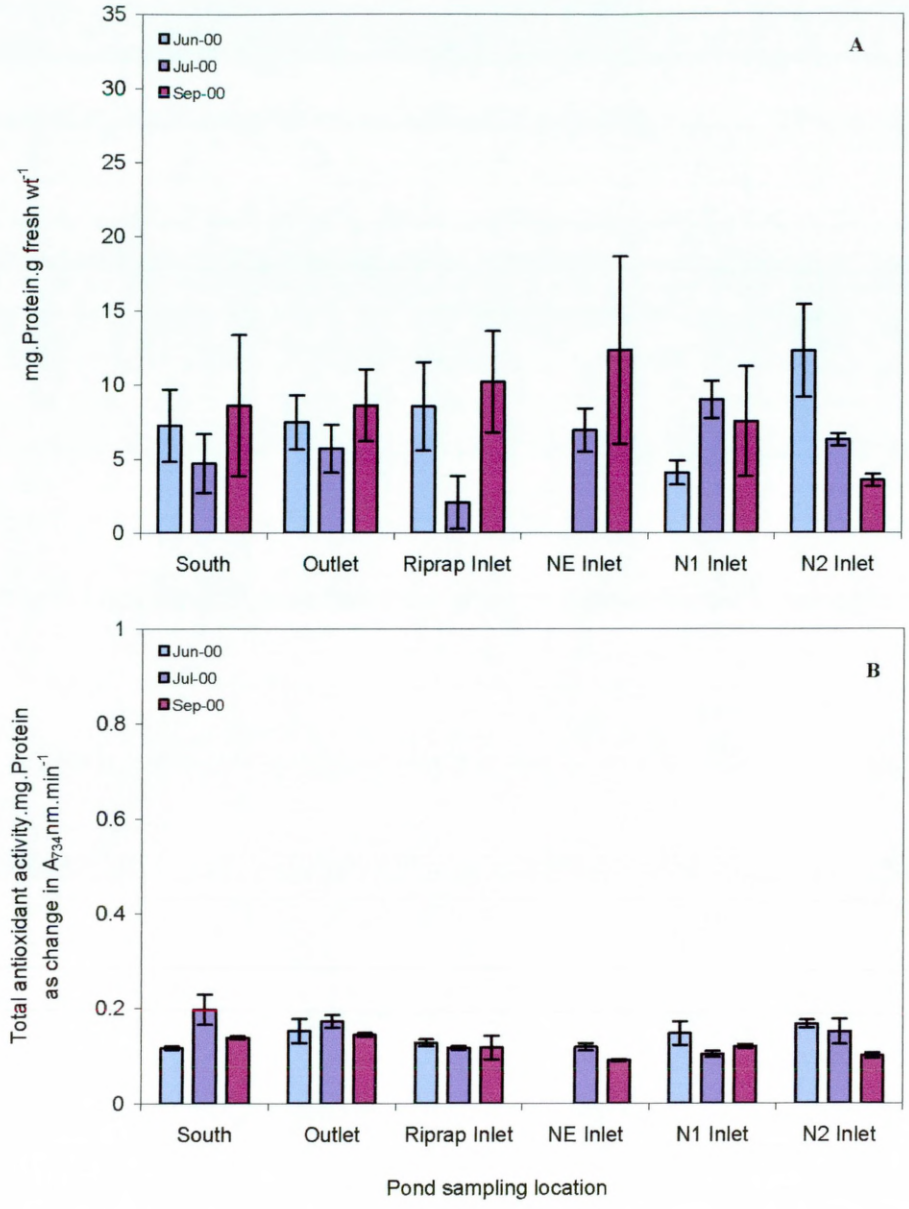


Figure 3.1 Profiles of protein and total antioxidant status in *C. glomerata* samples from Linburn pond during June – Sept 2000.

A – Changes in Linburn pond protein levels of *C. glomerata* between sampling month and pond sampling location and B change in Linburn pond total antioxidant activity of *C. glomerata* between sampling month and pond sampling location.

n = 3, error terms are expressed as standard deviations.

3.3.2.2 Halbeath Pond

Significant differences were detected between mean protein levels depending on sampling month ($F_{3,106} = 3.47$; $p < 0.05$) and sampling year ($F_{1,106} = 162.71$; $p < 0.001$) when data were transformed to the power 0.3 and analysed using a general linear model of a two way ANOVA. Tukey's pairwise comparisons showed that *C. glomerata* samples collected during the month of June 2000 have significantly lower protein levels than samples collected during July, August and September 2000. Algal samples collected during June and September 2001 inclusive show significantly higher mean protein levels than corresponding samples collected during year 2000 ($p < 0.001$). There were no significant differences in mean protein levels and pond sampling location in either 2000 or 2001 algal samples ($F_{3,106} = 0.89$; $p > 0.05$) (Figure 3.2 A and Figure 3.3 B).

There was no significant difference in the mean total antioxidant activity amongst pond sampling month ($F_{3,106} = 1.32$; $p > 0.05$) or sampling year ($F_{1,106} = 1.48$; $p > 0.05$) when data were transformed to power 3 and analysed using a general linear model of a two way ANOVA. Significant differences were detected in the mean transformed antioxidant activity amongst pond sampling locations ($F_{3,106} = 13.71$; $p < 0.001$) (Figure 3.2 B and Figure 3.3 B) using a one way ANOVA. Tukey's pairwise comparison showed samples collected from inlet locations have significantly lower antioxidant activity than those collected from the outlet, east and north pond locations.

No significant Pearson correlation was detected between combined protein levels and transformed total antioxidant activity from year 2000 and 2001 *C. glomerata* samples ($r = 0.136$; $p > 0.05$).

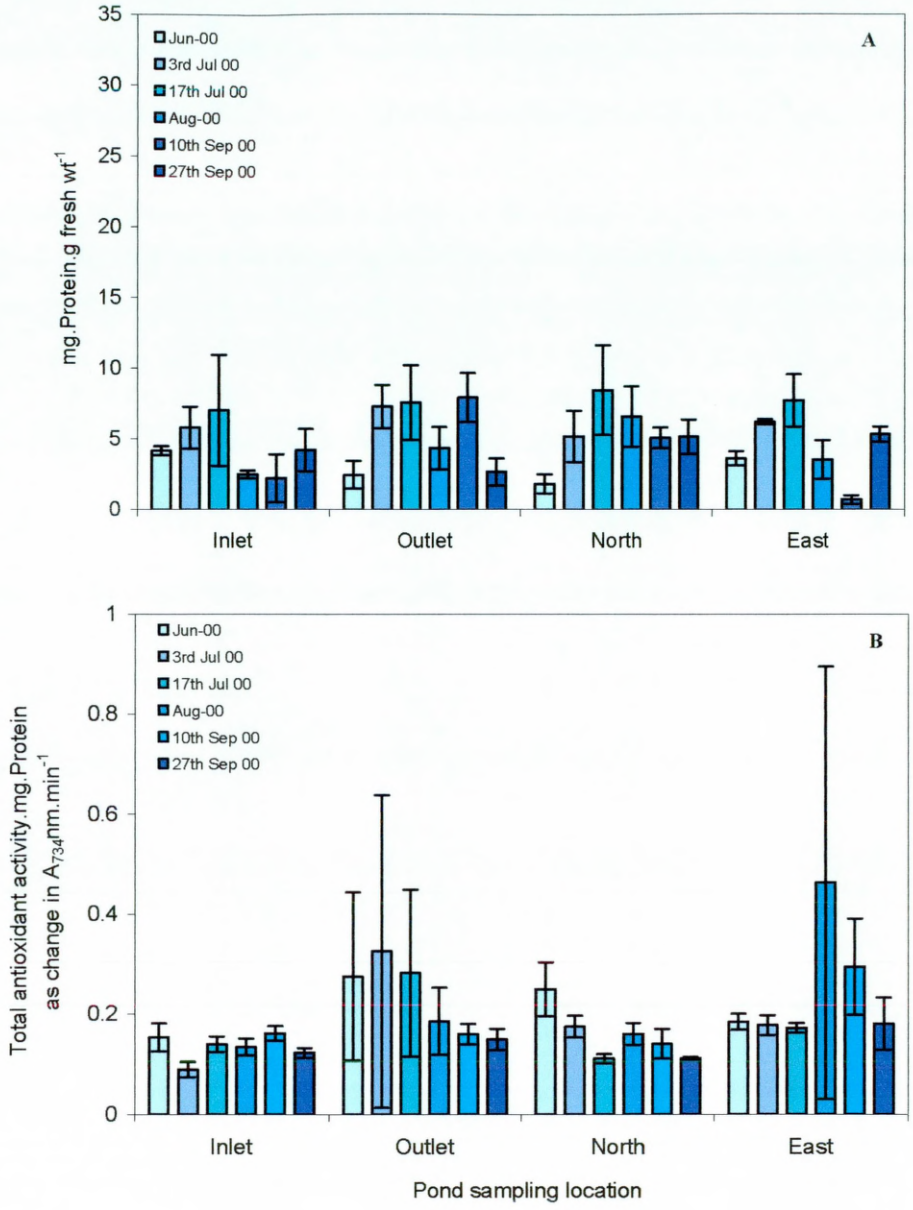


Figure 3.2 Profiles of total antioxidant activity and protein in *C. glomerata* sampled from Halbeath pond during June – Sept 2000.

A - Changes in Halbeath pond protein levels of *C. glomerata* between sampling month (Year 2000) and pond sampling location and B change in Halbeath pond total antioxidant activity of *C. glomerata* between sampling month (Year 2000) and pond sampling location.

n = 3, error terms are expressed as standard deviations

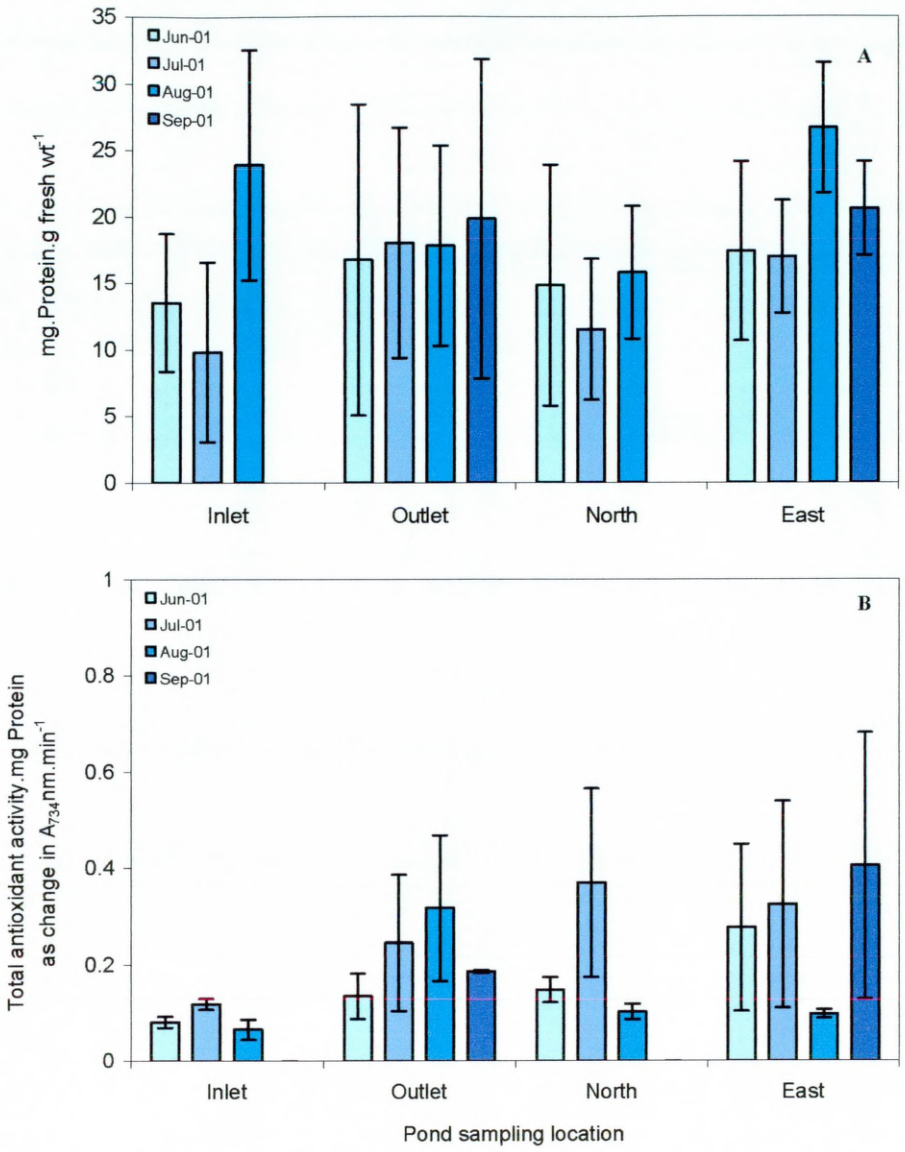


Figure 3.3 Profiles of total antioxidant activity and protein in *C. glomerata* samples from Halbeath pond during June – Sept 2001.

A - Changes in Halbeath pond protein levels of *C. glomerata* between sampling month (Year 2001) and pond sampling location and B change in Halbeath pond total antioxidant activity of *C. glomerata* between sampling month (Year 2001) and pond sampling location.

n = 3, error terms are expressed as standard deviations.

3.3.2.3 Pond 7

No significant differences or interactions [site and month interactions] ($F_{3,16} = 0.08$; $p > 0.05$) were detected in algal protein levels between sampling month ($F_{1,16} = 0.43$; $p > 0.05$) or sampling location ($F_{3,16} = 2.24$; $p > 0.05$) when data were analysed using a two way ANOVA (Figure 4.4 A).

Significant differences and interactions were detected between mean total antioxidant activity, sampling month ($F_{1,16} = 14.25$; $p < 0.01$) and pond sampling location ($F_{3,16} = 8.90$; $p < 0.01$) when data were analysed using a two way ANOVA. *C. glomerata* samples collected during the month of August 2000 show higher antioxidant activity compared with samples collected during July 2000 (Figure 3.4 B). In addition, outlet pond samples show increased antioxidant activity and those from south side location decreased antioxidant activity compared with both inlet site samples (north and south west) (Figure 3.4 B).

No significant Pearson correlations were detected between protein levels and total antioxidant activity in *C. glomerata* samples collected from Pond 7 ($r = 0.106$; $p > 0.05$).

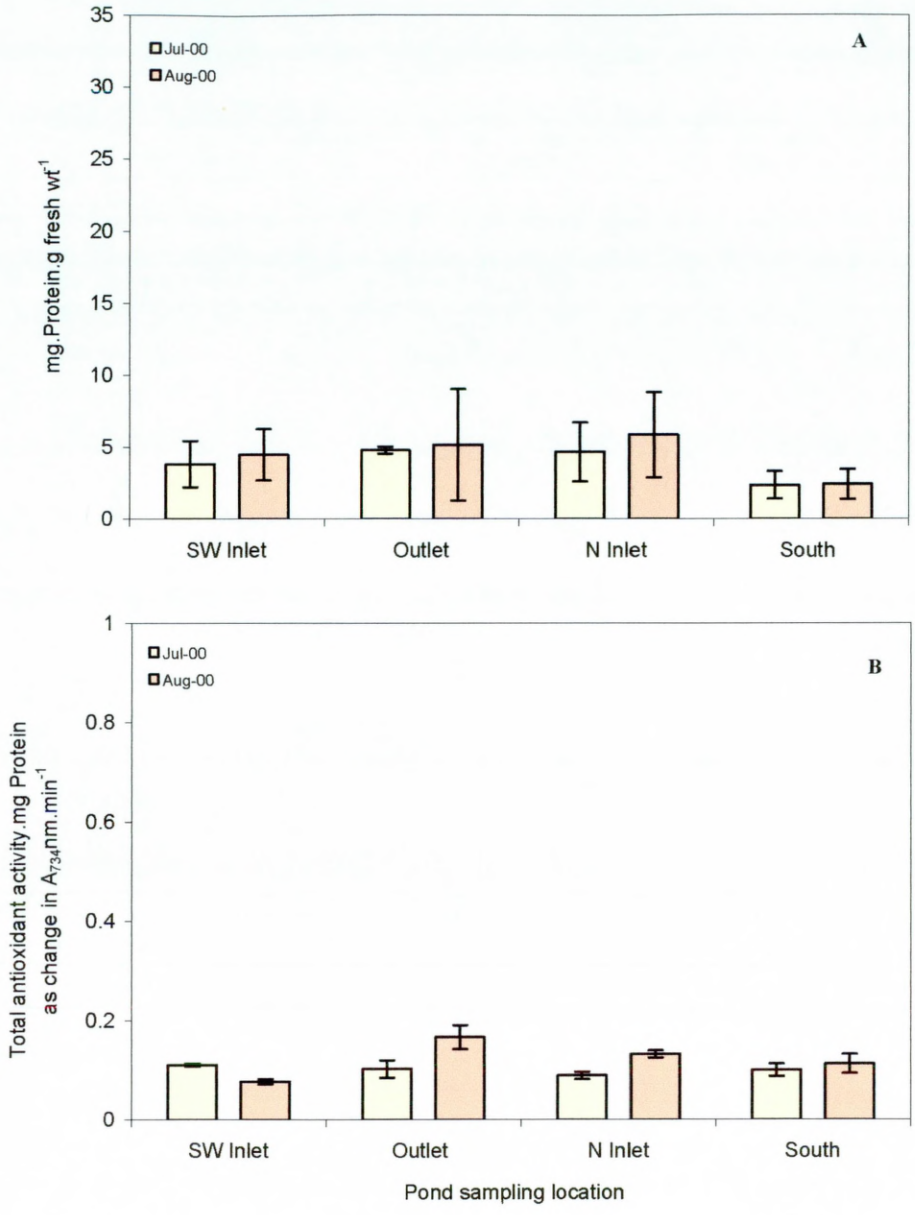


Figure 3.4 Profiles of total antioxidant status and protein in *C. glomerata* sampled from Pond 7 during July and August 2000.

A - Changes in Pond 7 protein levels of *C. glomerata* between sampling month and pond sampling location and B change in Pond 7 total antioxidant activity of *C. glomerata* between sampling month and pond sampling location.

n = 3, error terms are expressed as standard deviations.

3.4 Discussion

Due to their ecological importance in aquatic foodwebs and their ability to respond rapidly to a wide range of pollutants, algae are ideal indicator organisms that can provide useful early warning signals of deteriorating conditions in aquatic ecosystems. However, despite their importance in the water environment, algae are often ignored as indicators of aquatic ecosystem change due to a lack of standardized methods for monitoring with algae (McCormick & Cairns, 1994). This study has overcome the lack of monitoring methods by developing a simple, reliable and reproducible biochemical assay that uses algal stress responses as a marker of environmental change in urban ponds. Results so far, from this study demonstrate that this is a promising indicator assay and is therefore recommended for use in an environmental monitoring programme to determine the health status of many disturbed ecosystems using algae as the indicator species.

3.4.1 Extraction procedures and total antioxidant assay

Applying liquid nitrogen in combination with grinding and sonication (on ice) provided a reliable and robust technique for the complete extraction of algal intracellular material. Total antioxidant analysis performed on the algal supernatant obtained using this technique indicated that the antioxidant enzymes could be readily and reproducibly extracted from *C. glomerata*.

This was the first time that the total antioxidant assay had been applied to algae and the macroalga *C. glomerata*. The assay was assessed on its applicability to algae and its performance in terms of reproducibility, efficiency and fast through put of many samples. The assay proved itself to be reproducible by providing consistent results

within replicate samples and also applicable to algae as it revealed differences within the algal samples (3.3.2.1 – 3.3.2.3). This assay can be used as an indicator assay not only for macroalgae, but also for many biological samples from which the assay is derived (Miller *et al.*, 1996; Miller *et al.*, 1993; Re *et al.*, 1999) in an antioxidant programme to measure total antioxidant activity.

3.4.2 Protein and antioxidant enzymes

As previously highlighted in Chapter 1, reactive, toxic, oxygen species (ROS) are generated not only as a result of normal metabolic processes (Alscher *et al.*, 1997) but they are also stimulated by various environmental stresses (1.5). Exposure of plants to high light conditions (Foyer *et al.*, 1997), drought (Smirnoff, 1993), heavy metals (Weckx & Clijsters 1996), high salt concentration (Meneguzzo *et al.*, 1999) and extremes of temperature (Doke *et al.*, 1994) all induce toxic reactive oxygen species which can adversely affect vital functions such as photosynthesis, respiration, membrane leakage, senescence and susceptibility to various pathogens (Scandalios, 1997).

In addition to affecting vital functions, exposure to environmental stress can cause a change in algal protein content. Previous work on the effect of temperature in the cyanobacterium *Synechocystis* PCC 6803 by Rady *et al.* (1994) demonstrated that during colder temperatures a significant increase in antioxidant activity occurred with a concomitant decrease in protein content. This trend in increasing antioxidant activity with decreasing protein content was investigated for the alga *C. glomerata* and incorporated into the study to investigate whether a relationship exists between algal protein content and enhanced levels of total antioxidant activity.

Water quality and sediment data collected during the two year study (Chapter 2) reflects a pond system that contains fluctuating levels of environmental stressors similar to those discussed above. All of these stressors have the potential to generate ROS within *C. glomerata* where the intensity of attack depends on the severity of stress. A severe stress would generate a high level of ROS, consequently enhancing antioxidant production to combat the elevated ROS. Using the newly developed total antioxidant assay, it is possible to determine which *C. glomerata* samples have been exposed to more stress and it would be expected that these samples may have biochemical profiles that reflect stress impacts at the level of antioxidants. Subsequently, this assay was employed to determine if a relationship exists between enhanced antioxidant activity and pond location sites that are susceptible to maximal pollutant inputs (i.e. inlets) as compared to locations which have minimal pollutant input (i.e. outlets).

Despite fluctuating protein content between all samples in *C. glomerata* collected from Linburn pond and Pond 7 (Figure 3.1 A – Figure 3.4 A), statistical analysis revealed no significant differences in protein content and sampling month or location (Figure 3.5). Highly variable data suggests that algal stability was compromised either due to life cycle stages, abiotic limitations such as light, temperature and nutrients or low level ROS attack from biotic factors.

Algal growth can be influenced by nutrient (Dodds & Gudder, 1992), light and temperature limitations (Cox & Norton, 1994) all of which can have an affect on protein content. Data from Chapter 2 shows that a reduction in algal growth due to nutrient limitation and temperature (increases/decreases above normal levels) is unlikely since the ponds fell into the trophic category of mesotrophic-eutrophic range for nutrients (Table 2.2) and temperature was in the normal range during the algal growth season

(data not reported) (Surveys 1A – 3C). However, chlorophyll levels from the alga reveal chlorophyll *a/b* ratios of between 1.9:1 and 3.3:1 (Table 2.1). Under normal conditions, *C. glomerata* chlorophyll *a/b* should be in a ratio of 2.6:1 (Larkum & Barrett, 1983). Fluctuating chlorophyll *a/b* ratios indicate either reduced light penetration due to turbidity, or high light intensity (Reger & Krauss, 1970), both of these factors have the potential to affect algal growth and subsequently protein content.

Life cycle stages are also an essential component of algal growth rates (Appendix 2A). *C. glomerata* although collected during the main growth season in this study, would be undergoing various life cycles changes such as reproduction and towards the end of the growth season senescence, both of which have the ability to influence growth rates and protein content. This is reflected in protein data collected from Halbeath pond during 2000 and 2001 where it is highly likely that life cycle changes caused the increase in protein content of algal samples collected from 2001 than samples from 2000 (Figure 3.3 A & 3.6). The difference in protein content between years was unlikely to be due to an improvement in pond quality with respect to pollutant inputs, as data from Chapter 2 indicate that water quality was similar for both years (Figures 2.4 – 2.10). Therefore, the difference in protein content between years is more likely to be as a result of life cycle changes and turbidity impacts than pond water quality improvement.

Results from this study indicate that algal stability and growth was probably affected by a combination of biotic, abiotic and life cycle factors, which in turn enhanced antioxidant enzyme activities, as was the case in algae collected from Linburn pond and Pond 7 during spring-summer 2000. Previous studies investigating the effects on the alga *Tetraselmis gracilis* of exposure to Cd and the effects of metal mixtures (Hg, Cd, Pb and Cu) on *Gonyaulax polyedra* (Okamoto & Colepicolo, 1998) and *Chlorella*

vulgaris to UV-B exposure (Malanga & Puntarulo, 1995) all resulted in a prominent increase in antioxidant activity. Samples collected from Linburn pond during June and July 2000 and from locations outlet and south side had enhanced antioxidant activity indicating ROS attack (Figure 3.1 B & 3.5). In addition, Pond 7 also demonstrated enhanced antioxidant activity particularly during August and from outlet locations (Figure 3.4 B & 3.5). Enhanced levels of antioxidant production to combat low-medium ROS attack may in turn reduce the intensity of damage within the samples, without adversely interfering with growth rates and hence protein content. Excessive ROS attack results in an antioxidant system that cannot cope with the bombardment of free radicals, which ultimately can impair growth and reduce protein content as was seen in studies carried out by Rady *et al.*, 1994.

The hypothesis initially proposed in this study, that enhanced antioxidant activity could be anticipated at the inlets due to maximal pollutant input does not hold true for Halbeath pond as the results indicate lower total antioxidant activity at the pond inlet than the other sampling locations (Figure 3.2 B, 3.3 B & 3.6). Total antioxidant activity results from Linburn pond and Pond 7 also concur with this conclusion where enhanced activity was detected at the outlets and south side locations (minimal pollutant inputs) (Figure 3.5). However, the reduced antioxidant activities of algae collected from pond inlets may also indicate excessive stress and as a result antioxidant production may be compromised, the ("antioxidants used up") reducing antioxidant activity. It is expected that the inlets would have a higher load of xenobiotic pollutants e.g. organics, heavy metals which are not necessarily detected using water quality testing. Therefore at a biochemical level, algae may be more sensitive to microchanges in pollutant inputs, thus giving rise to the antioxidant results obtained in this study.

3.5 Conclusions

The total antioxidant assay was successfully employed for the investigation of stress in the alga *C. glomerata* collected from urban ponds. The assay proved to be reproducible providing a rapid throughput of many biological samples and therefore it is highly recommended that this assay be considered in an environmental monitoring programme to determine the health status of disturbed ecosystems using algae as the indicator species.

Algal stability and growth were affected in this study probably due to a combination of biotic, abiotic and life cycle changes and turbidity impacts. Samples collected from Linburn pond during June and July 2000 and from locations outlet and south side show enhanced antioxidant activity indicating ROS attack (Figure 3.5). In addition, Pond 7 also demonstrated enhanced antioxidant activity particularly during August and from outlet locations (Figure 3.5). Enhanced levels of antioxidant production may reduce the intensity of damage within the samples without adversely interfering with growth rates and hence protein content. However, there was no significant difference in algal protein content from both Linburn pond and Pond 7 samples (Figure 3.5). Furthermore, reduced antioxidant activities were detected at Halbeath pond inlet location (Figure 3.6) indicating exposure to excessive stress and as a result antioxidant production may be compromised thus reducing antioxidant activity. Moreover, significant differences in yearly protein content for Halbeath pond samples (Figure 3.6) were also detected however, this is more likely to be due to life cycle changes and turbidity impacts than an improvement in pond water quality. It is therefore concluded that the antioxidant enzyme data obtained in this study, indicates that at a biochemical level, algae may be

more sensitive to microchanges in pollutant inputs than could be detected using water quality testing.

Chapter 4 will investigate specific antioxidant responses of *C. glomerata* to environmental stress (biotic and abiotic) and relate the responses of the alga to pond health.

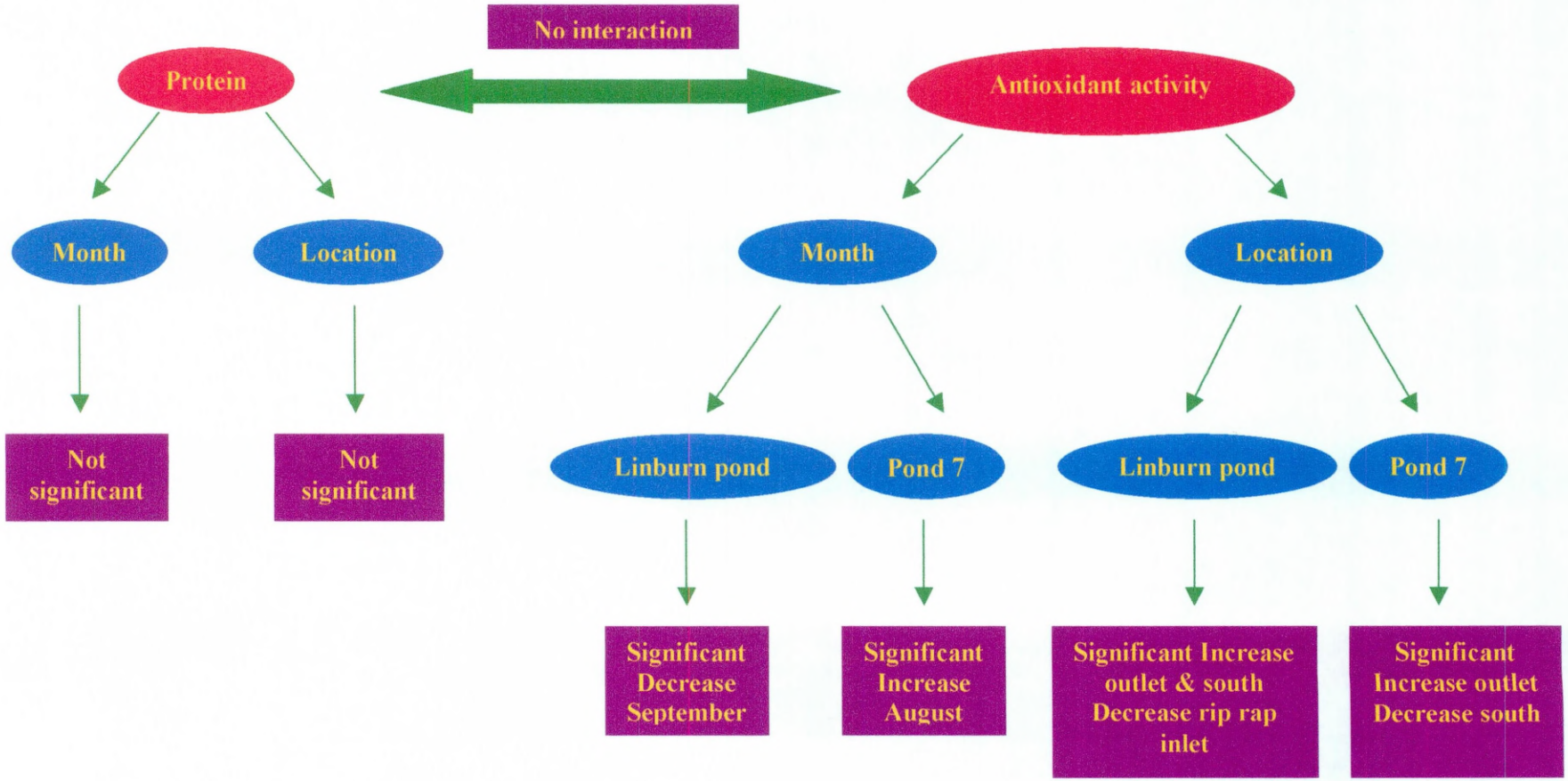


Figure 3.5 Statistical differences and interactions in protein content and antioxidant activities between sampling month and pond locations for Linburn pond and Pond 7 (Zar, 1996).

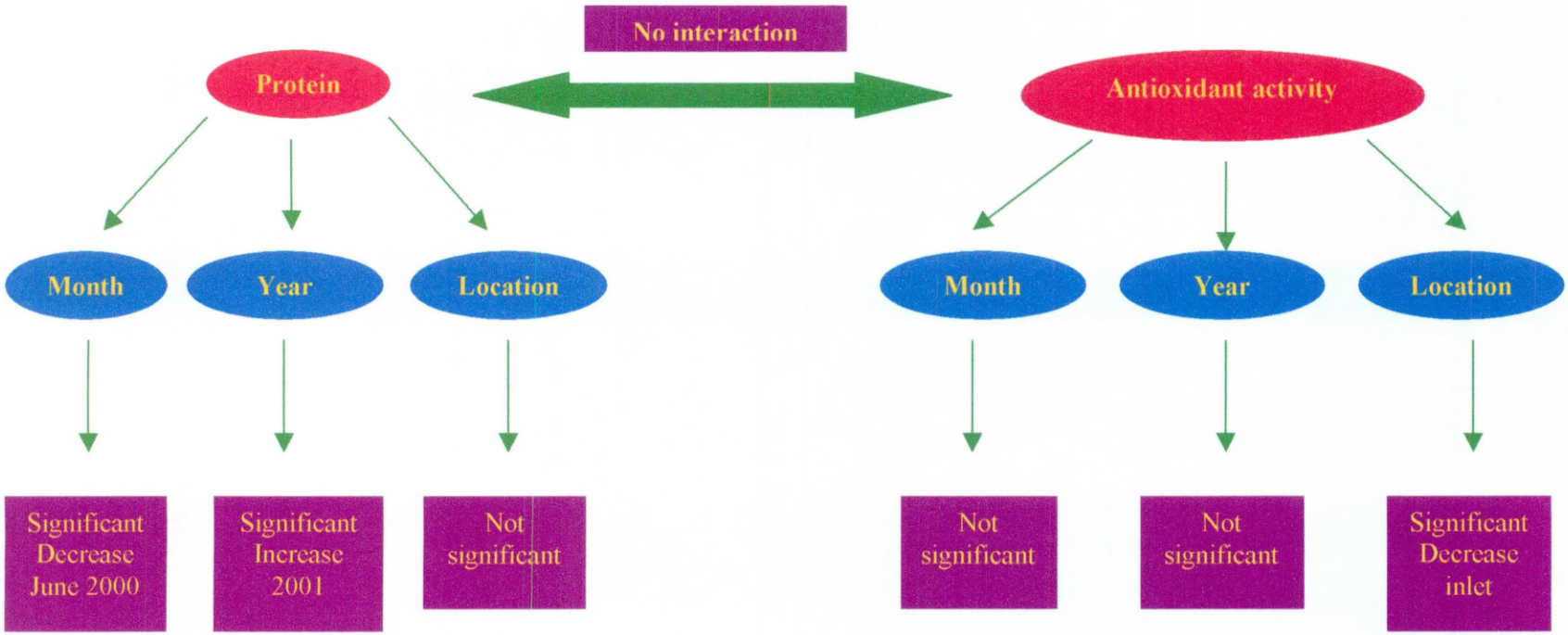


Figure 3.6 Statistical differences and interactions in protein content and antioxidant activities between sampling month, pond location and year sampled for Halbeath pond (Zar, 1996).

Chapter 4 INVESTIGATION OF SPECIFIC ANTIOXIDANT ACTIVITY IN *C. GLOMERATA* FROM URBAN PONDS

4. Introduction

Previous research has indicated that biotic and abiotic stress give rise to increased ROS levels (Alscher *et al.*, 1997; Doke *et al.*, 1994). Cellular responses to ROS involve the increased biosynthesis of antioxidant and ROS-decomposing enzymes (Bartosz, 1997). Increased antioxidant activity has been observed in certain strains of algae in response to stresses including: drought, temperature, UV-B exposure, heavy metals and herbicide exposure (Smirnoff, 1993; Rady *et al.*, 1994; Malanga & Puntarulo 1995; Okamoto *et al.*, 1996; Vartak & Bhargava 1999). The interaction between antioxidants and mechanisms for the control of deleterious ROS is likely to be an important factor for the survival and reproduction of the macroalga *C. glomerata* exposed to stress in urban ponds. In the context of this chapter, the specific antioxidant activity profiles of *C. glomerata* will be assessed and the algal specific activities coupled with total antioxidant activity data (Chapter 3) thus providing a more detailed and in depth understanding of algal and pond health with respect to pollutant inputs. This investigation will also highlight particular pond 'hot spot' locations for stress responses. In terms of applied research, understanding the basis of protective responses to urban xenobiotics may assist the identification of algal species that have phytoremediation potential.

4.1 Antioxidants

Due to the wide-ranging distribution of algae and exposure to a variety of both biotic and abiotic stresses, they have evolved extensive antioxidant defence mechanisms to

combat the danger posed by the presence of reactive oxygen species (1.5.1 & 1.5.2). These antioxidant defences consist of both enzymatic and non-enzymatic mechanisms that act either dependently or independently and function to remove ROS or reduce ROS production (Karpinski *et al.*, 2000).

The non-enzymatic antioxidant group consisting of carotenoid pigments form the first line of defence against free radical production in chloroplasts where they protect the photosynthetic apparatus from excess light by energy dissipation (Mallick & Mohn, 2000). In addition, carotenoids also quench singlet oxygen ($^1O_2$) providing a protective pathway in non-photosynthetic membrane systems (Benson, 1990). Furthermore, vitamin E has been found to be an effective scavenger of free radical attack by preventing oxidative damage to the lipids of cellular membranes (Alscher *et al.*, 1997). Vitamin E reacts with and destroys the reactive forms of oxygen, protecting unsaturated fatty acids from oxidation (Mallick & Mohn, 2000).

Enzymatic antioxidants confer protection by removing ROS and their associated non-radical oxygen species such as hydrogen peroxide. Superoxide dismutase (SOD) catalyses the conversion of superoxide ($O_2^{\cdot -}$) to hydrogen peroxide (H_2O_2) and oxygen (O_2) (Halliwell, 1982). Reduced glutathione (coupled with ascorbate recycling) including catalase and several different peroxidases catalyses the breakdown of hydrogen peroxide (Halliwell, 1982; Asada & Takahashi, 1987). Removing hydrogen peroxide can prevent peroxides becoming toxic to cells and reduces H_2O_2 involvement in Haber-Weiss/Fenton chemistry. Reduced glutathione (GSH) acts as an important redox buffer in tissues, conferring protection by protecting oxygen-sensitive enzymes and susceptible protein thiol (SH) groups from oxidative damage by providing a

preferential substrate for S-H oxidation with the production of oxidised glutathione (GSSG) and water (Halliwell, 1982; Packer 1984; Alscher *et al.*, 1997).

4.1.1 Superoxide dismutase

Superoxide dismutase (SOD) consists of a group of three metalloisoenzymes characterised by different metal moieties (Cu-ZN SOD, Mn-SOD and Fe-SOD). The Cu-Zn SOD is predominantly located in eukaryotic organisms where it is inactivated by H_2O_2 (Salin, 1987). In plants, this enzyme is the most abundant of the three isoenzymes and is predominantly associated with the chloroplast (Mallick & Mohn, 2000). However, this form of the enzyme is absent in prokaryotic and eukaryotic algae with the exception of a few members of the Charophyceae family (Lumsden *et al.*, 1977). Mn-SOD is located in the mitochondria in plants (Salin, 1987). Fe-SOD is located in prokaryotic organisms and the plastids of some plants where it is inactivated by H_2O_2 (Salin, 1987; Blum & Fridovich, 1985). In plant tissues, forms of SOD are generally located in the chloroplast due to their susceptibility to oxygen toxicity and in non-photosynthetic tissues it is found in the cytoplasm and mitochondrial membranes (Halliwell, 1982). SOD antioxidant enzymes catalyse the reaction of the superoxide radical ($O_2^{\cdot-}$) to oxygen (O_2) and hydrogen peroxide (H_2O_2). The reaction sequence allows for both the scavenging of the reactive oxygen species and the regeneration of the oxidised catalyst (Benson, 1990).

$$SOD_{ox} + O_2^{\cdot-} \longrightarrow SOD_{red} + O_2$$

$$2H^+ + SOD_{red} + O_2^{\cdot-} \longrightarrow SOD_{ox} + H_2O_2$$

Although SOD removes the potentially damaging superoxide radical ($O_2^{\cdot-}$), the reaction product, H_2O_2 can also be highly toxic to the cell. This can be exacerbated due to

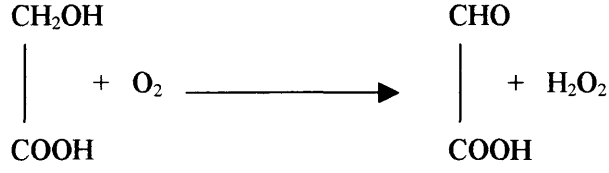
potential inhibition of SOD by H_2O_2 (Blum & Fridovich, 1985) subsequently, hydrogen peroxide can react with $O_2^{\cdot-}$ in the presence of transition metals (Fe^{2+}/Fe^{3+}) to produce the highly reactive and most damaging radical, the hydroxyl radical ($^{\cdot}OH$) (Chapter 1 and Chapter 5). There are a number of antioxidants that can either directly or indirectly remove H_2O_2, including the antioxidant enzymes catalase, peroxidase and glutathione reductase (Halliwell, 1974; 1982).

4.1.2 Catalase

Catalase is a tetrameric, haeme-containing enzyme found abundantly in aerobic eukaryotic organisms, where it is absent in chloroplasts (Van Ginkel & Brown, 1978) and associated with the peroxisome in plant tissues (Halliwell, 1982). The function of catalase is to catalyse the breakdown of potentially damaging H_2O_2 to H_2O and O_2.

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

In plants, H_2O_2 is produced not only as a product of the SOD reaction, but also during photorespiration, whereby glycolate oxidase catalyses the O_2-dependent production of glyoxylate from glycolate in plant peroxisomes (Halliwell, 1974).



Catalase protects the cell against radical injury by limiting H_2O_2 toxicity in the cells and preventing the compound from re-entering toxic oxidative reactions (Fenton chemistry). Furthermore, the removal of H_2O_2 generated in the chloroplast as a result of dismutation

of superoxide is achieved by the activity of peroxidases and is essential in avoiding the inhibition of CO_2-fixation cycle enzymes (Asada, 1992; Halliwell, 1974; 1982; Kaiser, 1976).

4.1.3 Peroxidase

Peroxidase is a ubiquitous enzyme, widely distributed in plants, mammals, fungi, prokaryotes and green algae (Asada, 1992; Ishikawa *et al.*, 1993). In plants, guaiacol peroxidases participate in many physiological processes such as the biosynthesis of lignin and auxin degradation (Halliwell, 1982; Salin, 1987; Asada, 1992). In contrast, ascorbate peroxidase distributed in higher plants, chlorophyte algae and *Euglena gracilis* (Appendix 2B) functions to eliminate hydrogen peroxide (Asada, 1992; Asada *et al.*, 1993; Amako *et al.*, 1994) and plays an essential role in scavenging hydrogen peroxide in chloroplasts (Asada & Takahashi, 1987). Immediate scavenging of H_2O_2 in chloroplasts is indispensable for maintenance of photosynthetic activity during illumination in higher plants (Asada, 1992). Peroxidase enzymes therefore protect cell membranes and membrane-bound enzymes from damage and loss of cell integrity (Ishikawa *et al.*, 1993).

$$\text{Substrate-H}_2 + \text{H}_2\text{O}_2 \xrightarrow{\text{peroxidase}} \text{Substrate} + 2\text{H}_2\text{O}$$

Research by Shigoeka *et al.* (1980) and Takeda *et al.* (1993) have demonstrated that there may be considerable difference in the susceptibility of photosynthesis to H_2O_2 between algae and higher plants as *Euglena gracilis* lacks catalase and ascorbate peroxidase activity in the cytosol and *Chlamydomonas reinhardtii* has low activity of ascorbate peroxidase in the chloroplasts compared to higher plants.

4.1.4 Sulphydryl groups

Sulphydryl groups can be split into non-protein thiol groups represented by very low molecular weight compounds such as the amino acid cysteine or glutathione, or high molecular weight protein thiols (protein bound sulphydryls) (Faure & Lafond, 1995). Reduced glutathione (GSH) acts as an important redox buffer, conferring protection by protecting oxygen-sensitive enzymes and susceptible protein thiol groups from oxidative damage by providing a preferential substrate for S-H oxidation (Halliwell, 1982; Packer 1984; Alscher, 1989).

$$2GSH + {}^{1}/_{2}O_2 \longrightarrow GSSG + H_2O$$

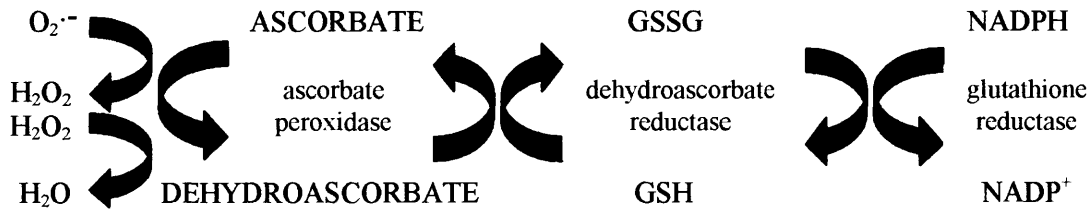
Accumulation of oxidised glutathione (GSSG), can be toxic to the cells (Halliwell, 1982); however, the enzyme glutathione reductase catalyses its degradation and recycles the protective GSH molecule.

$$GSSG + NADPH + H^+ \xrightarrow[\text{reductase}]{\text{glutathione}} 2GSH + NADP^+$$

Oxidation of protein sulphydryl groups by ozone leads to membrane damage within plant cells causing a large decrease in cellular protein SH content (Chevrier *et al.*, 1988). However, the level of cellular non-protein SH compounds remains relatively stable suggesting that ozone reacts preferentially with the sulphydryl groups of membrane proteins (Chevrier *et al.*, 1988) and thus confers protection by acting as a preferential target for attack by ROS.

4.1.5 Glutathione reductase

Glutathione reductase enzyme maintains reduced intracellular glutathione (GSH) levels that are required for the reduction of oxidised protein thiol groups (Goldberg & Spooner, 1983). Within the chloroplast, GSH removes H_2O_2 coupled with ascorbate recycling where ascorbate peroxidase scavenges H_2O_2 reducing its involvement in Haber-Weiss/Fenton reactions therefore limiting the production of the highly toxic hydroxyl radical (Halliwell, 1982). Excessive levels of oxidised glutathione (GSSG) produced as a consequence of ascorbate recycling can prove toxic to the cell and therefore for most cellular functions glutathione must be available in its reduced form (GSH) (Mallick & Mohn, 2000).



A third function involving GSH concerns the detoxification of heavy metals and xenobiotic compounds (Potters *et al.*, 2002). Glutathione (GSH) acts as a substrate for the multifunctional antioxidant enzyme glutathione-s-transferase (GST). GST catalyses the conjugation of GSH with potentially damaging xenobiotics (Marrs, 1996) and can reduce damaging lipid peroxides to non-toxic hydroxides (Habig *et al.*, 1974). The enzymes covalently link GSH with electrophiles neutralizing the electrophilic sites of xenobiotics and rendering them more water soluble and less toxic to cells (Simons & Vander Jagt, 1977).

4.2 Materials and Methods

4.2.1 *C. glomerata* and extraction procedure

C. glomerata samples were collected from strategic areas within Linburn pond. Halbeath pond and Pond 7 during spring – summer months (see 3.2.1). *C. glomerata* samples were processed and extracted as described in 3.2.2.1 and 3.2.2.5.

4.2.2 Superoxide dismutase assay

SOD catalyses the dismutation of superoxide ($O_2^{\cdot-}$) producing hydrogen peroxide (H_2O_2) and oxygen (O_2) (1.5.2). Superoxide dismutase activity was determined in the algal supernatant, after extraction by sonication and liquid nitrogen (3.2.2.5) (Benson & Roubelakis-Angelakis 1992; 1994).

Superoxide dismutase (SOD) activity was determined spectrophotometrically by monitoring the inhibition of the reduction of nitrotetrazolium blue (NBT) at 25°C by superoxide radicals (produced by light mediated generation from riboflavin and methionine) (Beuchamp & Fridovich, 1971). Stock solutions (1-4) were prepared in advance (Table 5.1). Stocks 1-3 were protected from the light as they are light sensitive and all stocks were refrigerated (4°C). Each stock was kept for 1 week only to permit consistent results between samples and replicates.

Table 4.1 SOD stock solutions

Stock no.	Compound	Quantity
1	Riboflavin	4mg/100ml dH_20
2	NBT	40.9mg/50ml dH_20
3	Methionine	0.746g/50ml dH_20
4	Wing buffer pH 7.8	3.4g KH_2PO_4/500ml dH_20
	Containing $1x10^{-4}$ M EDTA*	4.36g K_2HPO_4/500ml dH_20

*6.72mg EDTA/200ml Wing buffer

A reaction mixture of stock solutions was prepared in sufficient quantity to permit all samples and replicates to be performed from the same batch of buffer. The proportions of the different stocks are as follows: Riboflavin (Stock 1) 0.3ml; NBT (Stock 2) 0.1ml; Methionine (Stock 3) 0.15ml and Wing Buffer (Stock 4) 100ml all combined together to form the assay reagent with which to perform the SOD assay.

2.98ml of combined assay reagent was added to 20µl of algal extract in a 10ml test tube. Control tubes (lacking algal extract), standards and sample tubes were distributed randomly within a rack and evenly exposed to the light of four 100W light bulbs for 20min. Following illumination, the lights were switched off and the tubes covered in a black material. The absorbance at 570nm was then measured for each tube and calculated as units of SOD as compared to a standard curve prepared from purified enzyme (Sigma) in the range 30-320U $SOD.ml^{-1}$ and was the mean of three replicates.

4.2.3 Catalase assay

Catalase enzymes catalyse the breakdown of hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2) (1.5.2) and can be measured by monitoring the rate of decrease in ultraviolet (UV) absorbance of hydrogen peroxide (H_2O_2) at 240nm (Aebi, 1983).

Catalase activity was determined in the algal supernatant, after extraction by sonication and liquid nitrogen (3.2.2.5) (Benson & Roubelakis-Angelakis 1992; 1994).

The reaction buffer of 50mM phosphate buffer (pH 7) was pre-prepared in advance as described in section 3.2.2.1 with the addition of 250µl of H_2O_2 per 100ml buffer immediately prior to use. 950µl of reaction buffer was dispensed into a quartz cuvette and 50µl of algal extract was then added, gently mixed. The rate of decrease in absorbance at 240nm was measured every 10sec over a period of 40sec and the absorbance change per min^{-1} was calculated. Samples were measured against a "blank" of phosphate buffer at 240nm. Standards were prepared in the range of 22 - 220U $catalase.ml^{-1}$ and enzyme activity expressed as absorbance change per $min.mg\ protein^{-1}$ and as the mean of three replicates.

4.2.4 Peroxidase assay

Peroxidase enzymes catalyse the oxidation of cellular components by either hydrogen peroxide or organic hydroperoxides (Asada, 1992).

$$AH_2 + H_2O_2\ (ROOH) \xrightarrow{\text{Peroxidase}} A + 2H_2O\ (ROH + H_2O)$$

Peroxidase activity in the extracted algal extracts (3.2.2.5) was measured spectrophotometrically by assaying for guaiacol specific peroxidase by measuring changes in absorbance due to the formation of guaiacol oxidation products at 470nm (Murphy & Huerta, 1990).

Potassium phosphate buffer (50 mM) was prepared in advance by mixing KH_2PO_4 (3.4g/500ml dH_20) and K_2HPO_4 (4.36g/500ml dH_20), pH 6.1. The reagent buffer was

prepared immediately prior to use and contained 50mM phosphate buffer (pH 6.1) with 16mM guaiacol (179µl/100ml buffer) and 2mM hydrogen peroxide (20.4µl/100ml buffer).

20µl algal extract (3.2.2.5) was added to 950µl reagent buffer in a cuvette and gently mixed. The absorbance change at 470nm was measured every 15sec over a period of 1min and the absorbance change per min^{-1} was calculated. Samples were measured against a "blank" of phosphate buffer at 470nm. Standards were prepared in the range of 0.29 - 2.9U peroxidase.ml^{-1} and enzyme activity expressed as absorbance change per min.mg $protein^{-1}$ and as the mean of three replicates.

4.2.5 Sulphydryl groups

Sulphydryl groups were determined for total and non-protein bound groups as described by Chevrier *et al.* (1988) based on the reduction of 5,5' –dithiobis-(2-nitrobenzioc acid) (DTNB) by SH groups to 2-nitro-5-marcaptobenzoic acid. The nitromercaptobenzoic acid anion has an intense yellow colour that can be used to measure SH groups (Sedlak & Lindsay, 1968). Sulphydryl groups were determined from Halbeath pond algal samples (Year 2000 and 2001), after extraction by sonication and liquid nitrogen applications (3.2.2.5) (Benson & Roubelakis-Angelakis 1992; 1994). Stock solutions (1–5) were prepared in advance.

Table 4.2 SH stock solutions

Stock no.	Compound	Quantity	Volume / Solvent
1	DTNB	0.3963g	100ml/methanol
2	Tris-HCl pH 8.2	21.12g Tris-HCl/20.04g Tris- base	300ml/dH_2O*
3	Tris-HCl pH 8.5	4.42g Tris-HCl/8.72g Tris-base	500ml/dH_2O*
4	Sodium dodecyl sulphate (SDS)	5g	100ml/dH_2O
5	Trichloroacetic acid (TCA)	5g	100ml/dH_2O

* pH Tris buffers in 10M NaOH

4.2.5.1 Total SH groups

C. glomerata was extracted as in section 3.2.2.5, with 8ml Tris-HCl (pH 8.2) used as the extraction buffer. 500µl 5% SDS (Stock 4) and 100µl DTNB (Stock 1) was then added to 2ml algal suspension and the mixture intermittently vortexed to ensure complete mixing during 20min incubation at room temperature. Ice-cold methanol (3ml) was added and the mixture centrifuged at 10,00rpm for 10min. The concentration of total SH groups were determined by measuring the absorbance at 412nm and comparing to a glutathione (GSH) standard curve prepared in the range 30 - 600µM.

4.2.5.2 Non – protein SH groups

C. glomerata was extracted as in section 3.2.2.5, with 8ml 5% (w/v) TCA (stock 5) used as the extraction buffer. The mixture was then vortexed, incubated on ice for 10min and finally centrifuged at 13,000rpm for 10 min. Cuvettes were prepared with 3ml Tris-HCl (Stock 3) and 100µl DTNB (Stock 1) to which was added 1ml of extracted algal sample. The mixture was gently mixed and incubated at room temperature for 2min. The concentration of non-protein bound SH groups was determined by measuring the

absorbance at 412nm and comparing this to a glutathione (GSH) standard curve prepared in the range 30 - 600μM.

4.2.6 Glutathione reductase assay

Glutathione reductase (GR) catalyses the splitting of oxidised glutathione (GSSG) back to reduced glutathione (GSH) coupled with the co-oxidation of nicotinamide-adenine dinucleotide phosphate (NADPH) to $NADP^+$ (1.5.2). The catalytic activity of glutathione reductase permits oxidation of NADPH to be monitored spectrophotometrically by a decrease in absorbance at 334nm as a marker of enzyme activity. Glutathione reductase activity was determined in the extracted algal supernatant (3.2.2.5) using the method as described by Goldberg & Spooner (1983).

Stock solutions (1 & 2) were prepared in advance (up to 1 week) or immediately prior for use in the assay (Stock 3 & 4).

Table 4.3 Glutathione reductase stock solutions

Stock no.	Compound	Quantity	Volume / Solvent
1	KH_2PO_4	16.33g	1L/dH_2O *
2	EDTA-$Na_2H_2.H_2O$	0.56g	100ml/dH_2O
3	GSSG	40mg	1ml/dH_2O
4	β-NADPH	8mg	1ml/1% (w/v) $NaHCO_3$ (in dH_2O)

*Phosphate buffer prepared by dissolving 16.33g KH_2PO_4 in 800 ml of dH_2O, adjusted to pH 7.2 using 1 M NaOH, diluting to a final volume of 1 L with dH_2O.

Cuvettes containing 2.6ml (Stock 1), 0.1ml (Stock 2) and 0.1ml (Stock 3) were prepared to which was added 0.1ml algal extract (3.2.2.5). After standing for 5 min, 0.05ml (Stock 4) was added, gently mixed and the change in absorbance was measured at 334nm every 15sec for a period of 1min to establish the absorbance change per min^{-1}.

A standard curve was prepared from purified glutathione reductase extract in the range 1.7 - 17U GR.ml^{-1} and enzyme activity expressed as absorbance change per min.mg protein^{-1} and as the mean of three replicates.

4.2.7 Glutathione-s-transferase assay

Glutathione transferases (GST) catalyse the conjugation of xenobiotic agents with the SH group of glutathione (GSH) therefore neutralizing xenobiotic electrophilic sites and rendering the products more water-soluble (Habig *et al.*, 1974). Glutathione-s-transferase activity was determined in the extracted algal supernatant (3.2.2.5) by measuring the increase in absorbance at 340nm due to the formation of the conjugate glutathione (GSH) (co-enzyme) with the artificial substrate 1-chloro-2,4-dinitrobenzene (CDNB) (Warholm *et al.*, 1985).

Table 4.4 GST stock solutions

Stock no.	Compound	Quantity	Volume / Solvent
1	Na_2HPO_4/EDTA-$Na_2H_2.H_2O$	1.42g/0.0372g	100 ml/dH_2O*
2	GSH	61.4mg	10 ml/dH_2O
3	CDNB	40.6mg	10 ml/95% Ethanol

*Sodium phosphate buffer (0.1M) containing 1mM EDTA, pH 6.5 with 1M HCl

850µl of buffer (Stock 1), 50µl GSH and 50µl CDNB was added to a 1ml cuvette and the mixture equilibrated at room temperature for 2min. Following reagent stabilization, 50µl of algal extract (3.2.2.5) was added to the cuvette and the increase in absorbance was measured every 15sec for a period of 1min to establish the absorbance change per min^{-1}. A standard curve was prepared from purified glutathione-s-transferase extract in the range 7.1 - 71U.ml^{-1} and enzyme activity expressed as absorbance change per min.mg protein^{-1} and as the mean of three replicates.

4.2.8 Data analysis using Minitab version 13

Results were analysed separately for each enzyme assay using ANOVA with month, location and year as factors. ANOVA assumes data within each level are normally distributed with the same standard deviation (Zar, 1996). Where the assumption of normality or equality of variance was not met data were analysed using a one way parametric test (distribution-free) such as Kruskal-Wallis or Chi-square (χ^2) (Sokal & Rohlf, 1981). Both tests make no assumptions about the distribution of the data (e.g. normality) where Kruskal – Wallis is an alternative to the parametric (distribution-dependant) ANOVA and Chi square tests the hypothesis of whether two samples are associated in some characteristic (Sokal & Rohlf, 1981), in the case of this chapter presence/absence of specific enzyme activity. All calculations were analysed using Minitab v.13 (Minitab, USA).

4.3 Results

To facilitate interpretation of differences in enzyme activity between each pond, the results have been presented on an enzyme/antioxidant basis. This allows variations between sampling month, sampling location and in the case of Halbeath pond year-to-year comparisons between each pond to be investigated. Samples where specific enzyme activity was not detected by the assay are omitted from the graphics section but are still included in the statistical analyses. Algal samples showing trace amounts of enzyme activity are included in the graphic results section but are at the limits of assay detection. Samples showing enzyme activity are included in the graphics sections and indicate that activity is present and can be easily detected by the assay. For clarification, in addition to the graphics, a summary table of specific enzyme activity for each pond sample is also included and categorised as trace activities and as presence or

absence of activities (Tables 4.11 - 4.13). Furthermore, a summary scheme showing significant differences in specific enzyme activity for each pond is also included and categorised as sampling month, pond location and year (Figures 4.13 – 4.17).

Table 4.5 Statistical analysis of superoxide dismutase activity in urban ponds

Pond	Factors	Method	Test statistic	p-value range	SOD activity
Linburn pond	Month	Kruskal-Wallis	$H = 3.30$	$p > 0.05$	n/s
	Location	Kruskal-Wallis	$H = 1.93$	$p > 0.05$	n/s
Pond 7	Month	Kruskal-Wallis	$H = 1.00$	$p > 0.05$	n/s
	Location	Kruskal-Wallis	$H = 3.00$	$p > 0.05$	n/s
Halbeath pond	Month	Kruskal-Wallis	$H = 1.95$	$p > 0.05$	n/s
	Location	Kruskal-Wallis	$H = 4.94$	$p > 0.05$	n/s
	Year	Chi-square	$\chi^2 (1) = 5.636$	$p < 0.05$	Increase year 2001

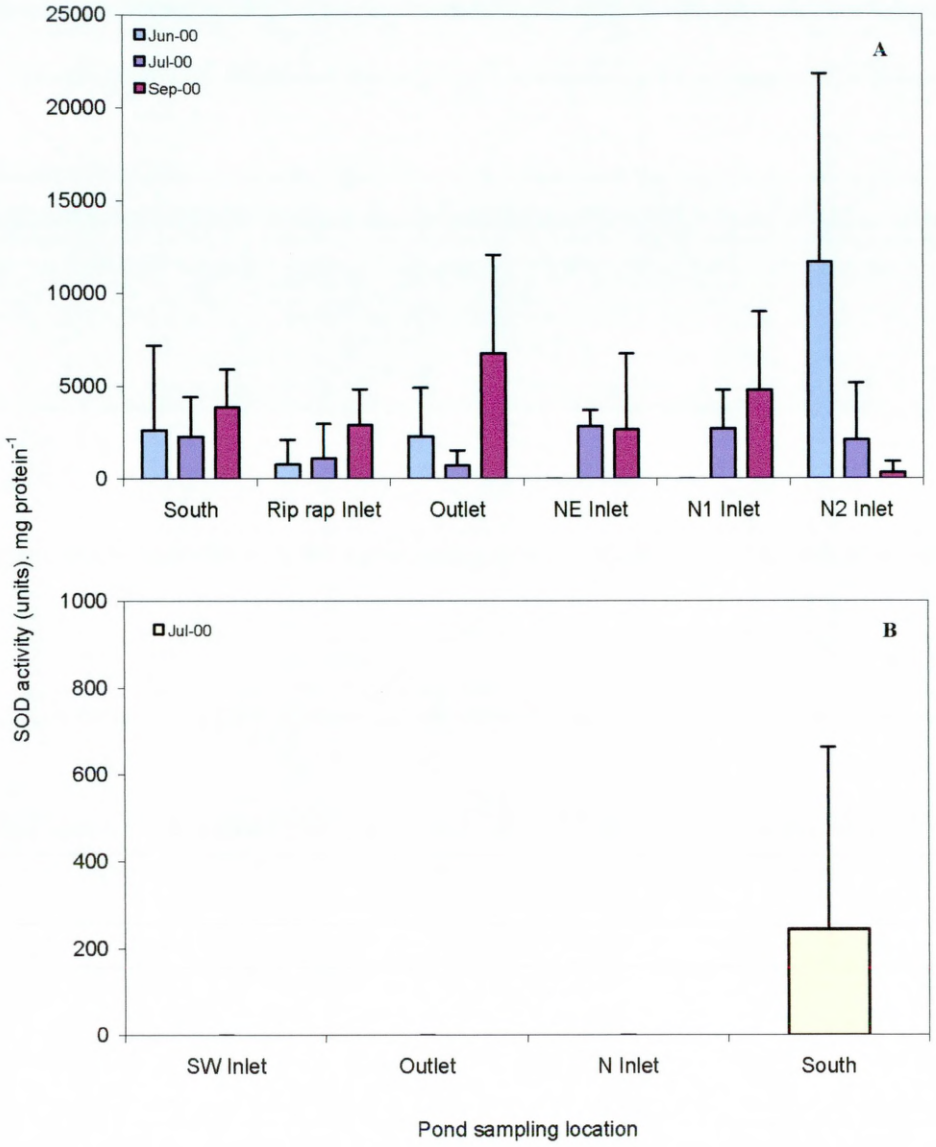


Figure 4.1 Profiles of SOD activity in *C. glomerata* sampled from Linburn pond during June-September 2000 and Pond 7 during July 2000.

A – Change in Linburn pond SOD activity for *C. glomerata* samples and B – change in Pond 7 SOD activity for *C. glomerata* samples (SOD activity absent from Pond 7 August 2000 samples (*Note the scale on the SOD axis changes between graphs).
n = 3, error terms are expressed as positive standard deviations.

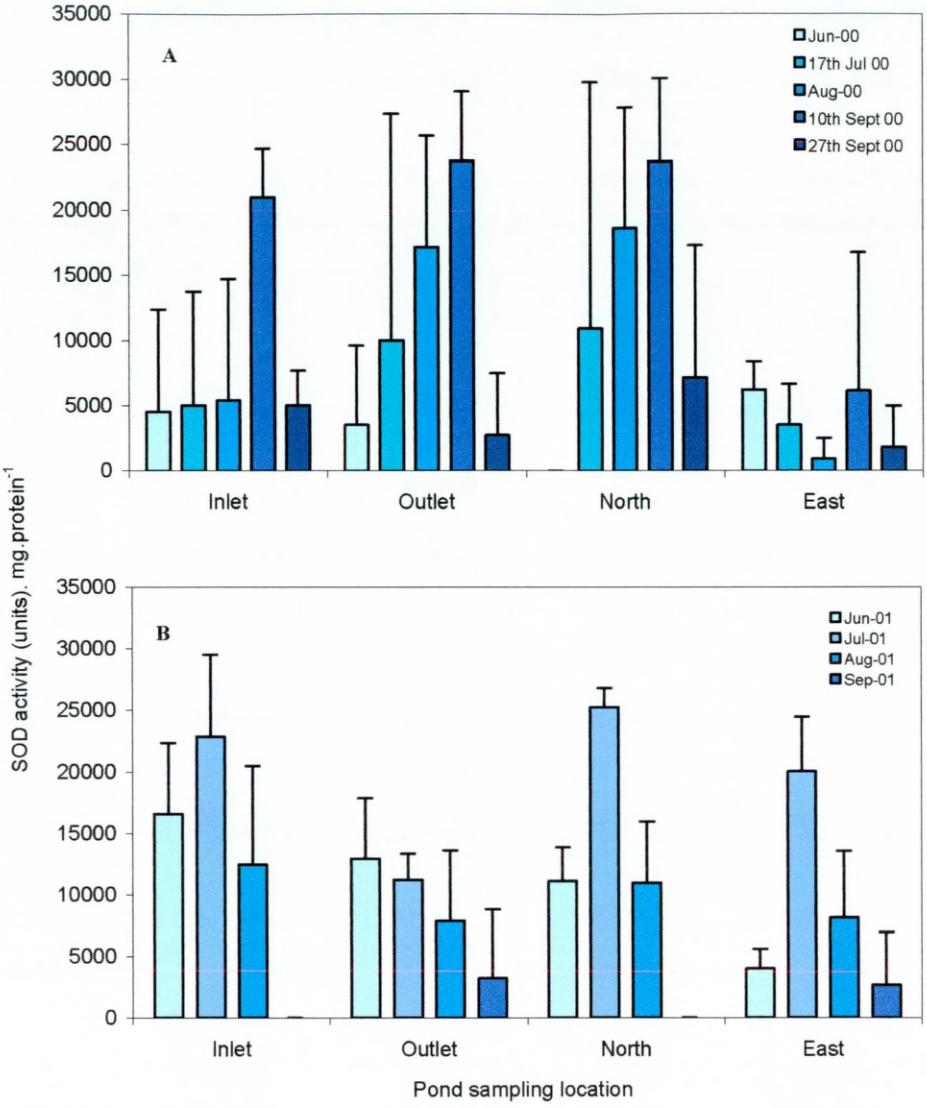


Figure 4.2 Profiles of SOD activity in *C. glomerata* sampled from Halbeath pond throughout June-September 2000 and 2001.

A - Change in Halbeath pond (year 2000) SOD activity for *C. glomerata* samples (*Note SOD activity absent from Halbeath 3rd July 2000 samples) and B – change in Halbeath pond (year 2001) SOD activity for *C. glomerata* samples.

n = 3, error terms are expressed as positive standard deviations.

Table 4.6 Statistical analysis of catalase activity in urban ponds

Pond	Factors	Method	Test statistic	p-value range	Catalase activity
Linburn pond	Month	Kruskal-Wallis	$H = 0.07$	$p > 0.05$	n/s
	Location	Kruskal-Wallis	$H = 1.24$	$p > 0.05$	n/s
Pond 7	Month	Two way ANOVA	$F_{(1,19)} = 14.85$	$p > 0.05$	n/s
	Location	Two way ANOVA	$F_{(3,19)} = 0.23$	$p < 0.001$	Increase July
Halbeath pond	Month	Kruskal-Wallis	$H = 13.42$	$p < 0.01$	Increase August (median = 1.20)
	Location	Kruskal-Wallis	$H = 2.13$	$p > 0.05$	n/s
	Year	Kruskal-Wallis	$H = 2.35$	$p > 0.05$	n/s

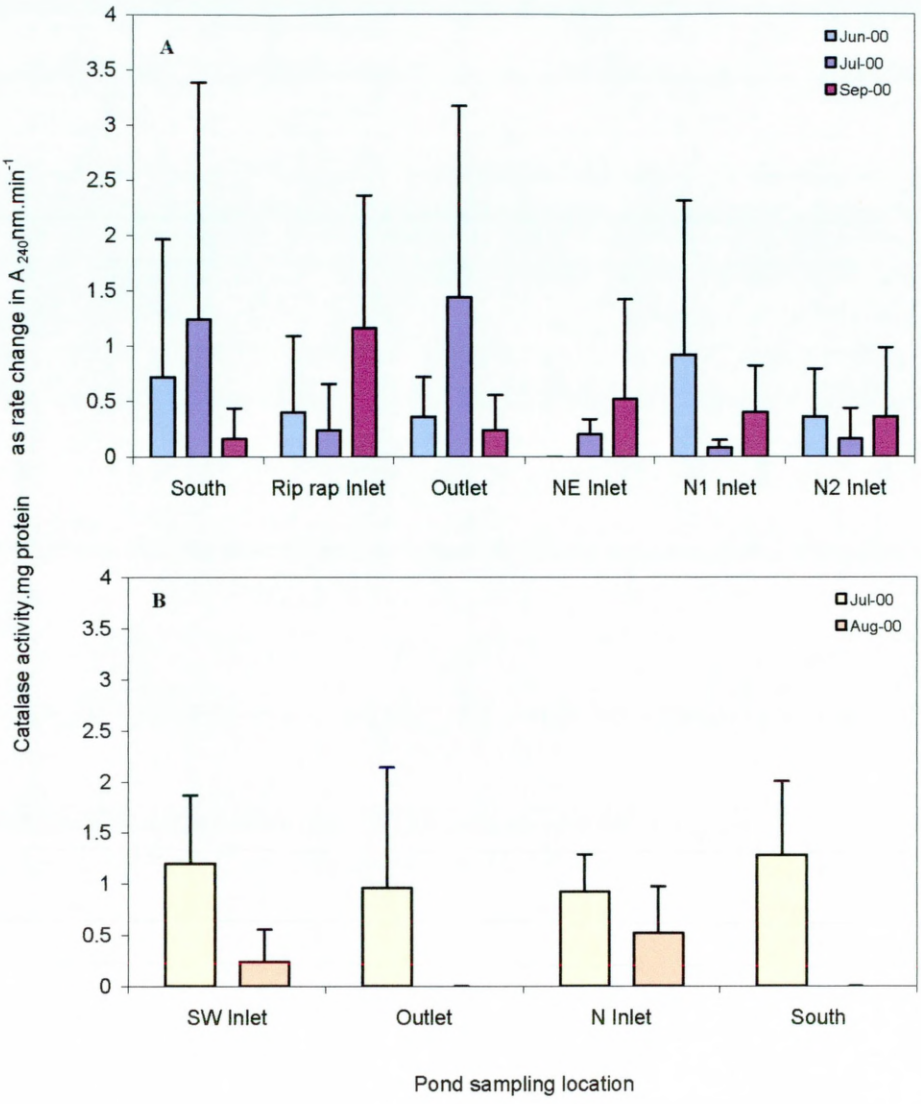


Figure 4.3 Profiles of catalase activity in *C. glomerata* sampled from Linburn pond during June-September 2000 and Pond 7 throughout July-August 2000.

A – Change in Linburn pond catalase activity for *C. glomerata* samples and B – change in Pond 7 catalase activity for *C. glomerata* samples.

n = 3, error terms are expressed as positive standard deviations.

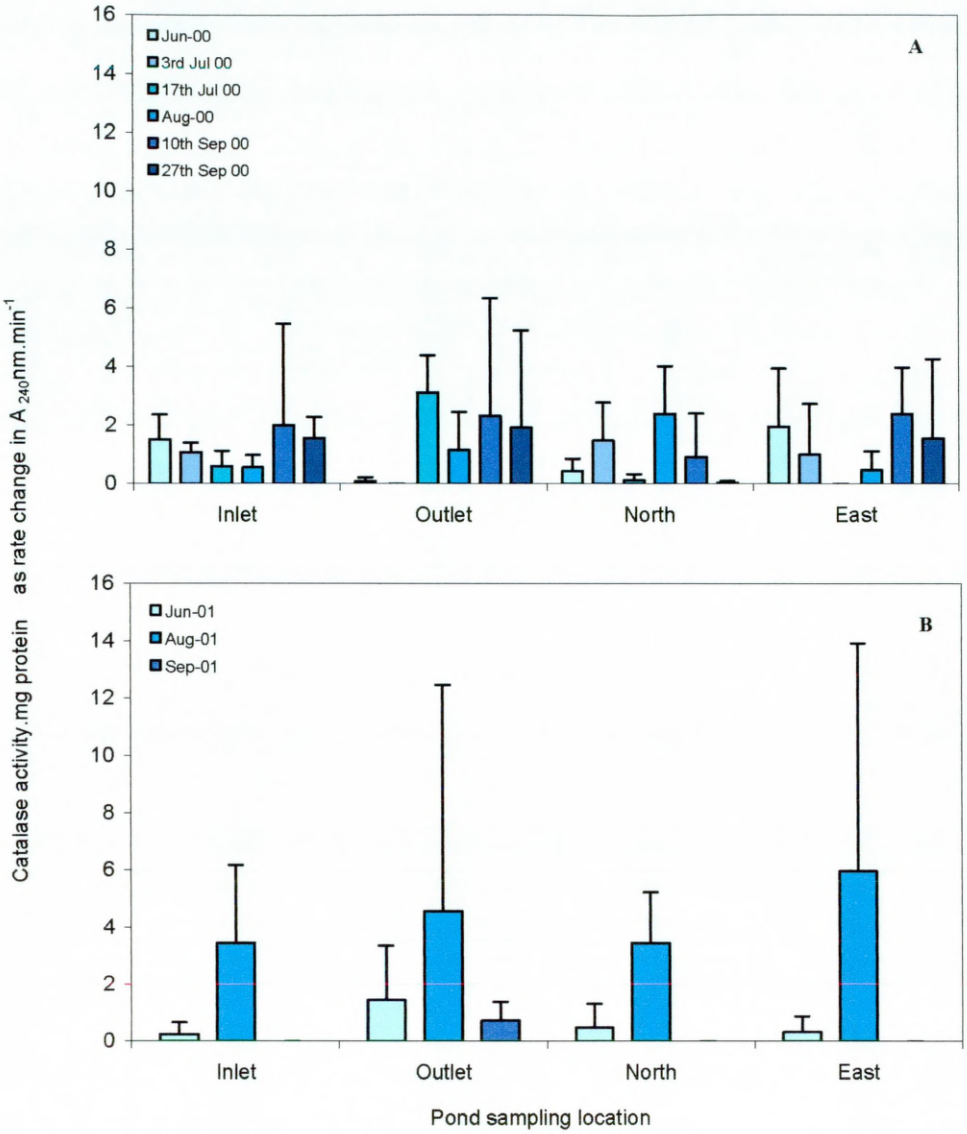


Figure 4.4 Profiles of catalase activity in *C. glomerata* sampled from Halbeath pond throughout June-September 2000 and 2001.

A - Change in Halbeath pond (year 2000) catalase activity for *C. glomerata* samples and B - change in Halbeath pond (year 2001) catalase activity for *C. glomerata* samples (*Note catalase activity absent from Halbeath July 2001 samples).

n = 3, error terms are expressed as positive standard deviations.

Table 4.7 Statistical analysis of peroxidase activity in urban ponds

Pond	Factors	Method	Test statistic	p-value range	Peroxidase activity
Linburn pond	Month	Kruskal-Wallis	$H = 19.84$	$p < 0.001$	Increase September (median = 0.0169)
	Location	Kruskal-Wallis	$H = 7.13$	$p > 0.05$	n/s
Pond 7	Month	Chi-square	$\chi^2 (1) = 20.308$	$p < 0.001$	Increase August
	Location	One way ANOVA	$F_{(3,19)} = 1.44$	$p > 0.05$	n/s
Halbeath pond	Month	Kruskal-Wallis	$H = 9.83$	$p < 0.05$	Increase July (median = 0.0542)
	Location	Kruskal-Wallis	$H = 1.20$	$p > 0.05$	n/s
	Year	Chi-square	$\chi^2 (1) = 36.277$	$p < 0.001$	Increase year 2000

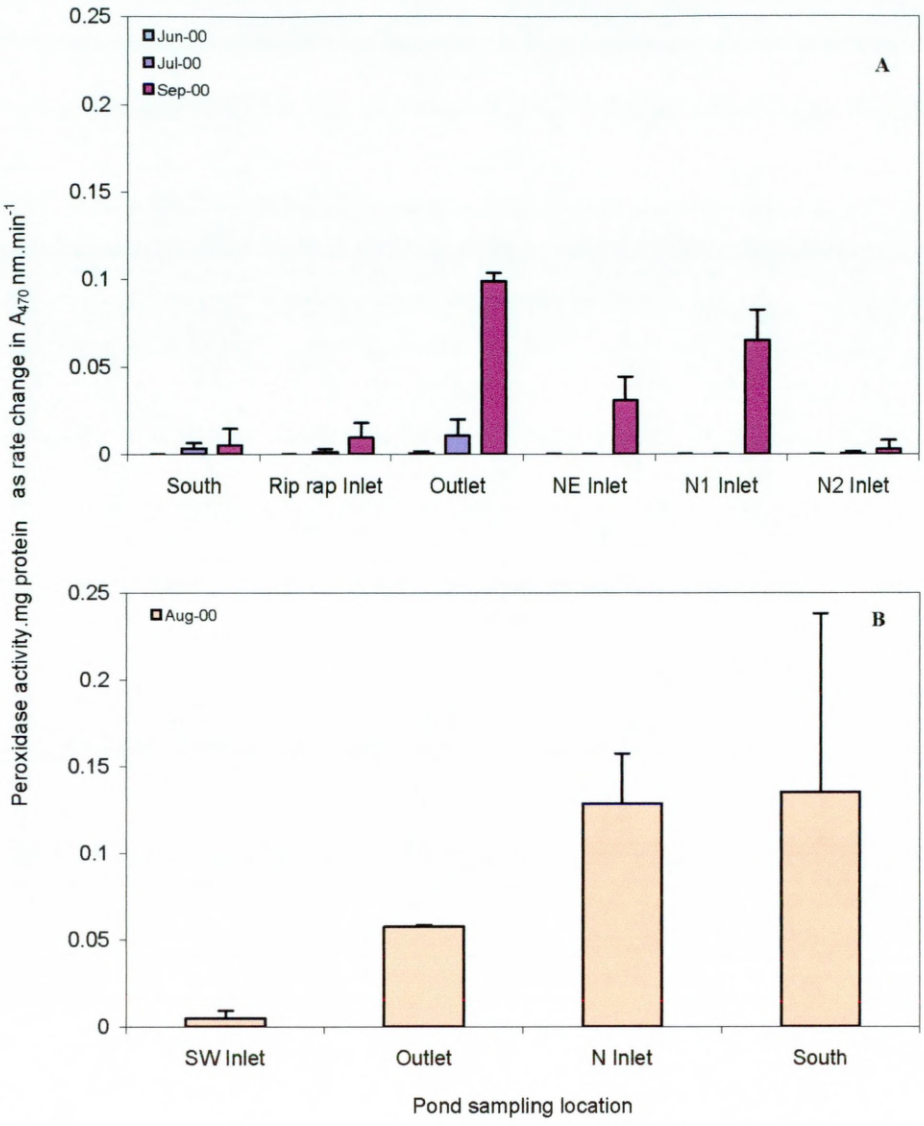


Figure 4.5 Profiles of peroxidase activity in *C. glomerata* sampled from Linburn pond during June-September 2000 and Pond 7 throughout July-August 2000.

A – Change in Linburn pond peroxidase activity for *C. glomerata* samples and B – change in Pond 7 peroxidase activity for *C. glomerata* samples (*Note peroxidase activity absent from Pond 7 July 2000 samples).

n = 3, error terms are expressed as positive standard deviations.

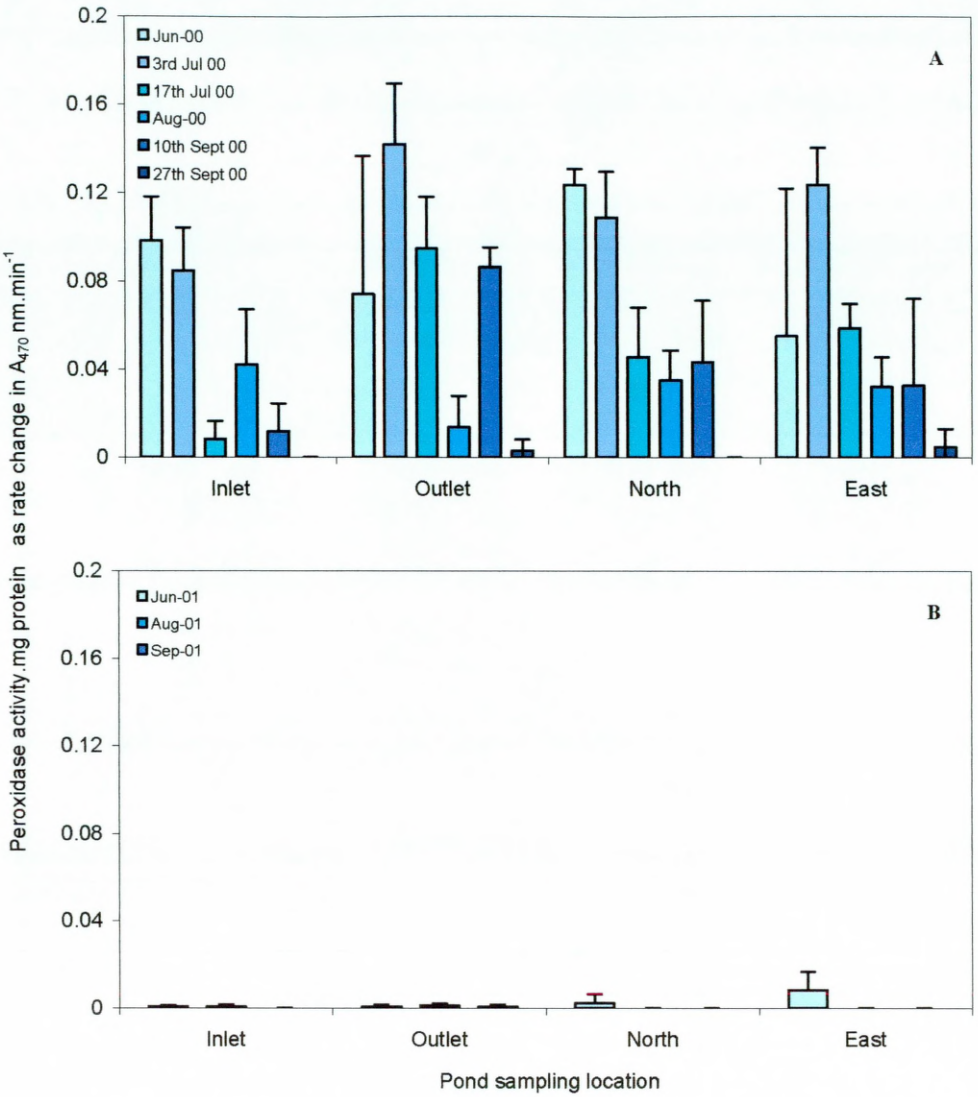


Figure 4.6 Profiles of peroxidase activity in *C. glomerata* sampled from Halbeath pond throughout June-September 2000 and 2001.

A – Change in Halbeath pond (year 2000) peroxidase activity for *C. glomerata* samples and B – change in Halbeath pond (year 2001) peroxidase activity for *C. glomerata* samples.

n = 3, error terms are expressed as positive standard deviations.

Table 4.8 Statistical analysis for sulphydryl content in urban ponds

Pond	SH groups	Factors	Method	Test statistic	p-value range	Non protein/protein SH groups
Halbeath pond	Non-protein	Month	Kruskal-Wallis	H = 3.28	p > 0.05	n/s
		Location	Kruskal-Wallis	H = 7.70	p > 0.05	n/s
		Year	Kruskal-Wallis	H = 20.62	p < 0.001	Increase non-protein year 2000 (median = 205.3)
Halbeath pond	Protein bound	Month	Kruskal-Wallis	H = 3.09	p > 0.05	n/s
		Location	Kruskal-Wallis	H = 9.12	p < 0.05	Lower protein SH Inlet (median = 123.6)
		Year	Kruskal-Wallis	H = 138.85	p < 0.001	Increase protein SH year 2000 (median = 421.647)

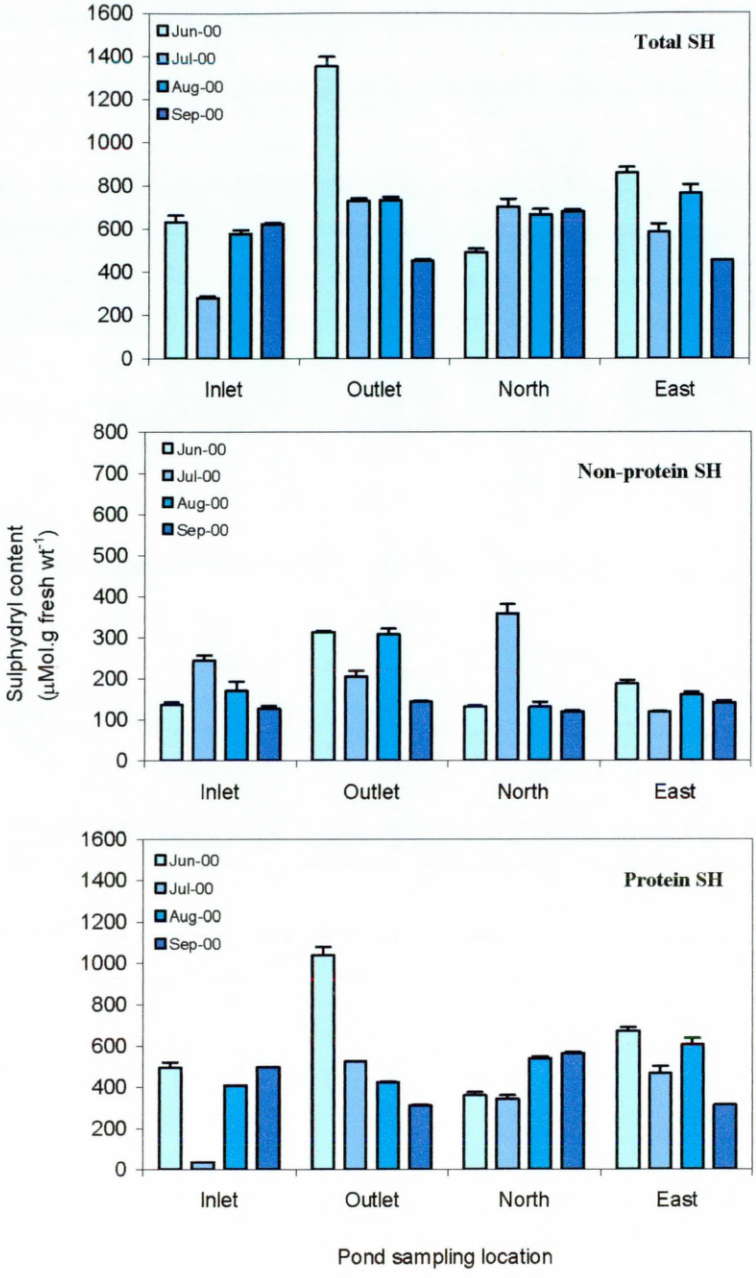


Figure 4.7 Profiles of sulphydryl concentration expressed as total, non-protein and protein sulphydryl groups in *C. glomerata* from Halbeath pond 2000.

Change in total sulphydryl, non-protein and protein sulphydryl content for *C. glomerata* samples (*Note the scale on the non-protein SH axis changes between graphs).
n = 9, error terms are expressed as positive standard deviations.

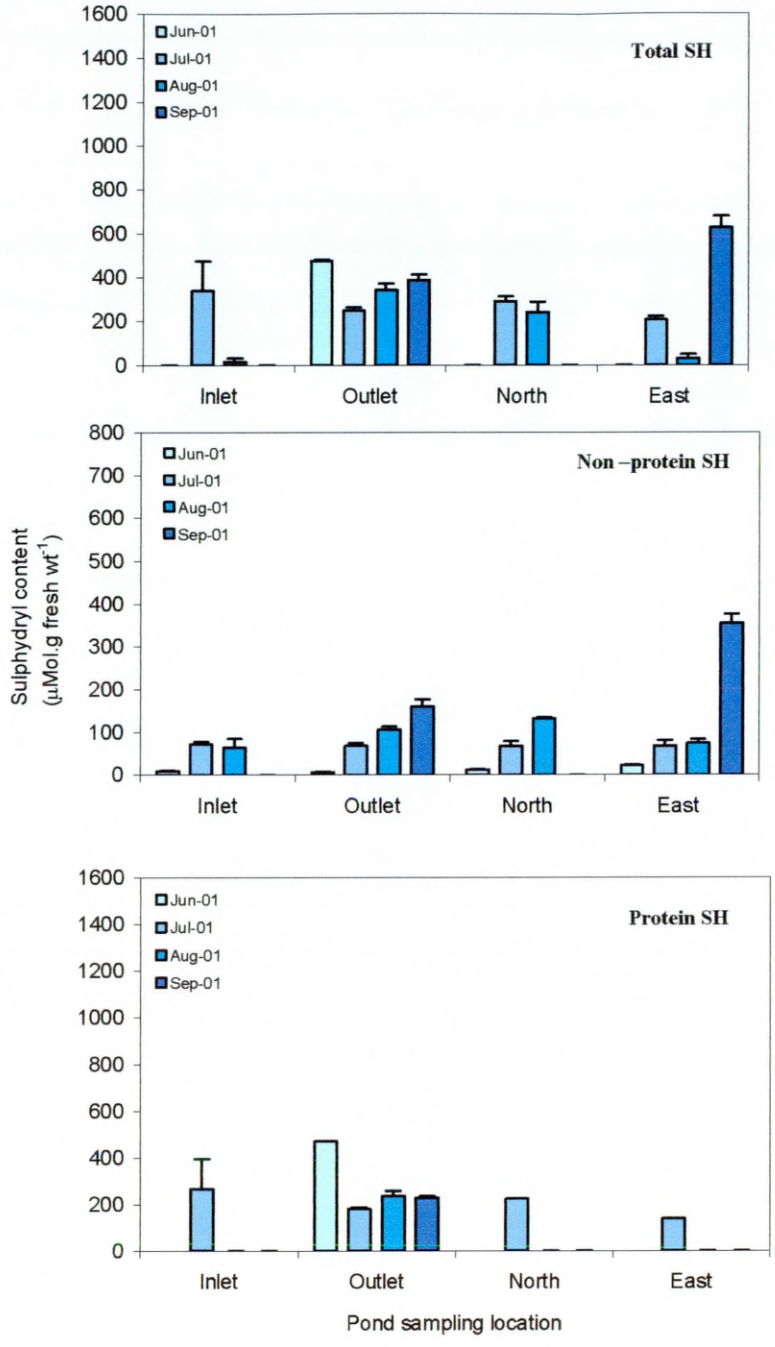


Figure 4.8 Profiles of sulphydryl concentration expressed as total, non-protein and protein sulphydryl groups in *C. glomerata* from Halbeath pond 2001.

Change in total sulphydryl, non-protein and protein sulphydryl content for *C. glomerata* samples (*Note the scale on the non-protein SH axis changes between graphs).

n = 9, error terms are expressed as positive standard deviations.

Table 4.9 Statistical analysis of glutathione reductase activity in urban ponds

Pond	Factors	Method	Test statistic	p-value range	Glutathione reductase activity
Linburn pond	Month	Kruskal-Wallis	$H = 0.38$	$p > 0.05$	n/s
	Location	Kruskal-Wallis	$H = 6.61$	$p > 0.05$	n/s
Pond 7	Month	Two way ANOVA	$F_{(1,19)} = 4.53$	$p < 0.05$	Increase July
	Location	Two way ANOVA	$F_{(3,19)} = 0.30$	$p > 0.05$	n/s
Halbeath pond	Month	Kruskal-Wallis	$H = 10.92$	$p < 0.05$	Decrease June (median = 0.04080)
	Location	Kruskal-Wallis	$H = 1.33$	$p > 0.05$	n/s
	Year	Kruskal-Wallis	$H = 19.66$	$p < 0.001$	Increase year 2000 (median = 0.13400)

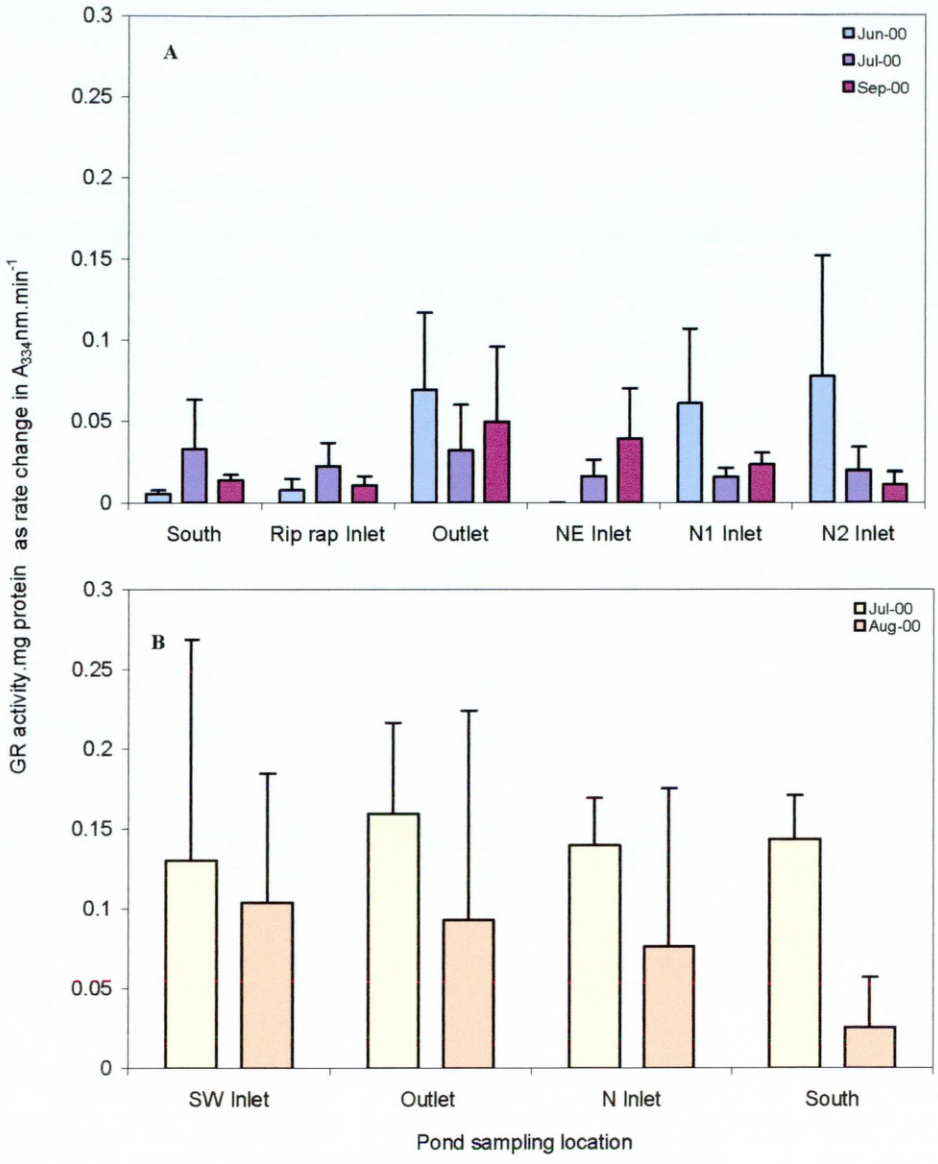


Figure 4.9 Profiles of glutathione reductase activity in *C. glomerata* sampled from Linburn pond during June-September 2000 and Pond 7 throughout July-August 2000.

A – Change in Linburn pond glutathione reductase activity for *C. glomerata* samples and B – change in Pond 7 glutathione reductase activity for *C. glomerata* samples.
n = 3, error terms are expressed as positive standard deviations.

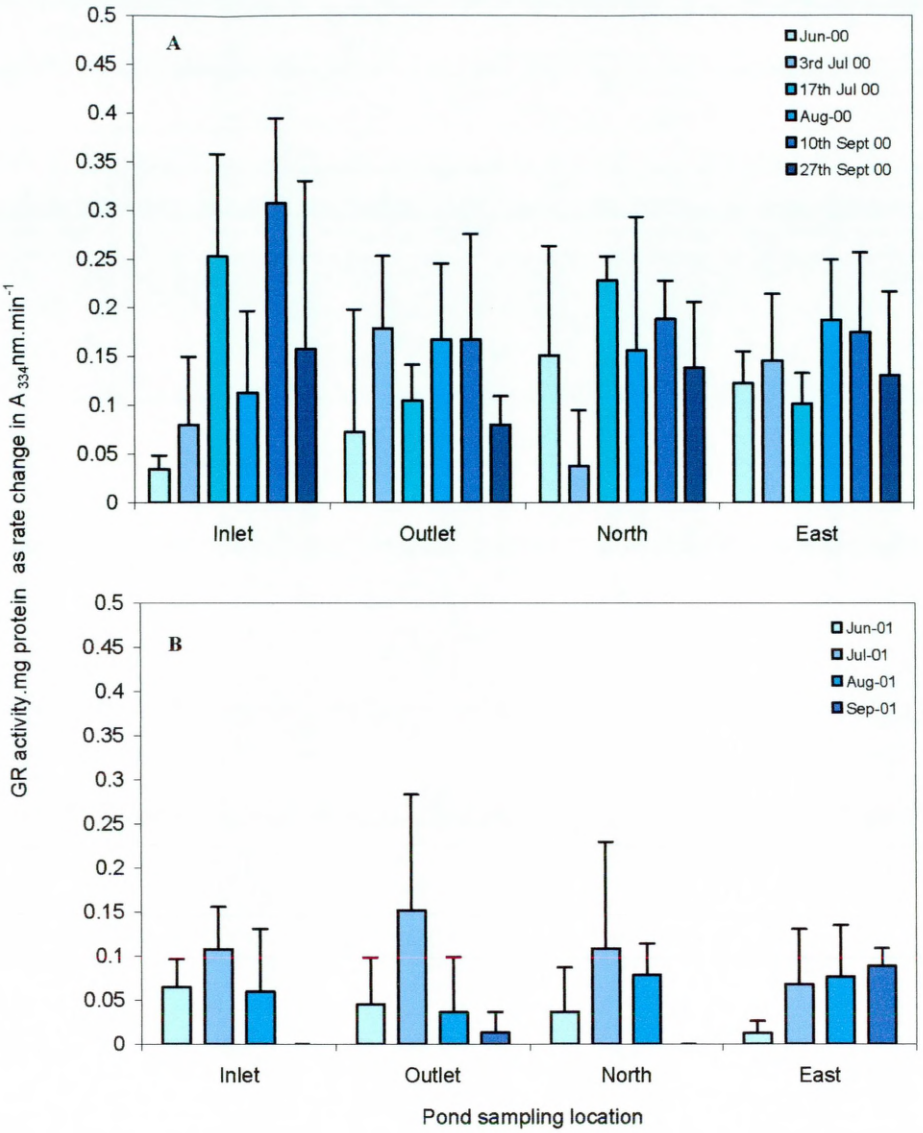


Figure 4.10 Profiles of glutathione reductase activity in *C. glomerata* sampled from Halbeath pond throughout June-September 2000 and 2001.

A – Change in Halbeath pond (year 2000) glutathione reductase activity for *C. glomerata* samples and B – change in Halbeath pond glutathione reductase activity (year 2001) for *C. glomerata* samples.

n = 3, error terms are expressed as positive standard deviations.

Table 4.10 Statistical analysis of glutathione-s-transferase activity in urban ponds

Pond	Factors	Method	Test statistic	p-value range	Glutathione-s-transferase activity
Linburn pond	Month	Kruskal-Wallis	$H = 2.27$	$p > 0.05$	n/s
	Location	Kruskal-Wallis	$H = 3.27$	$p > 0.05$	n/s
Pond 7	Month	Two way ANOVA	$F_{(1,19)} = 2.66$	$p > 0.05$	n/s
	Location	Two way ANOVA	$F_{(3,19)} = 1.70$	$p > 0.05$	n/s
Halbeath pond	Month	Kruskal-Wallis	$H = 1.51$	$p > 0.05$	n/s
	Location	Kruskal-Wallis	$H = 8.06$	$p < 0.05$	Increase East (median = 0.00320)
	Year	Kruskal-Wallis	$H = 12.30$	$p < 0.001$	Increase year 2000 (median = 0.00356)

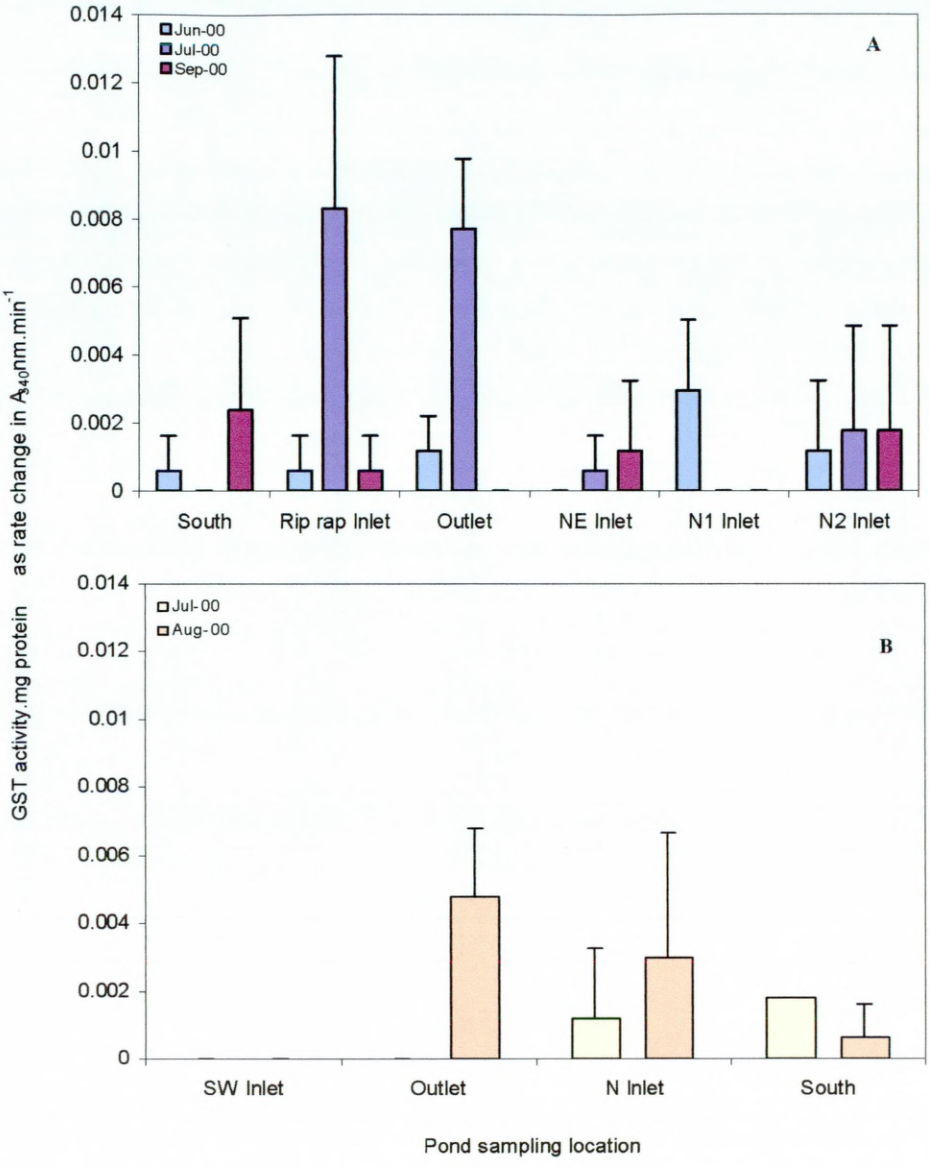


Figure 4.11 Profiles of glutathione-s-transferase activity in *C. glomerata* sampled from Linburn pond during June-September 2000 and Pond 7 throughout July-August 2000.

A – Change in Linburn pond glutathione-s-transferase activity for *C. glomerata* samples and B – change in Pond 7 glutathione-s-transferase activity for *C. glomerata* samples. n = 3, error terms are expressed as positive standard deviations.

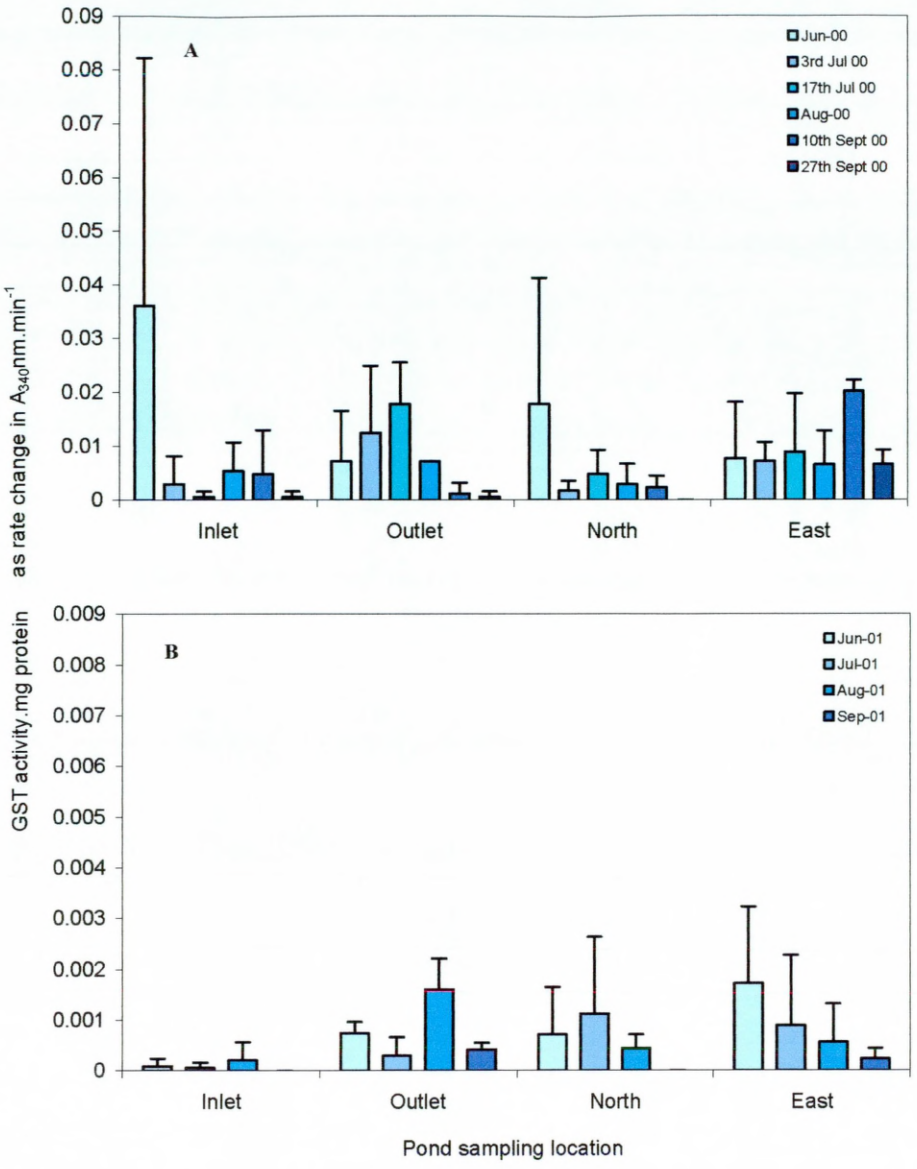


Figure 4.12 Profiles of glutathione-s-transferase activity in *C. glomerata* sampled from Halbeath pond throughout June-September 2000 and 2001.

A – Change in Halbeath pond (year 2000) glutathione-s-transferase activity for *C. glomerata* samples and B – change in Halbeath pond (year 2001) glutathione-s-transferase activity for *C. glomerata* samples. (*Note the scale on the GST axis changes between graphs)

n = 3, error terms are expressed as positive standard deviations.

Table 4.11 Summary of present/absent/trace specific enzyme activity for Linburn pond

Linburn Pond	Catalase activity						Peroxidase activity					
Month	South	RR Inlet	NE Inlet	N1 Inlet	N2 Inlet	Outlet	South	RR Inlet	NE Inlet	N1 Inlet	N2 Inlet	Outlet
Jun 2000	P	P	n/a	P	P	P	A	A	n/a	A	A	T
Jul 2000	P	P	P	P	P	P	T	T	A	A	T	P
Sep 2000	P	P	P	P	P	P	T	P	P	P	T	P

Linburn pond	SOD activity					
Month	South	RR Inlet	NE Inlet	N1 Inlet	N2 Inlet	Outlet
Jun 2000	P	P	-	A	P	P
Jul 2000	P	P	P	P	P	P
Sep 2000	P	P	P	P	P	P

Linburn Pond	Glutathione reductase activity						Glutathione-s-transferase activity					
Month	South	RR Inlet	NE Inlet	N1 Inlet	N2 Inlet	Outlet	South	RR Inlet	NE Inlet	N1 Inlet	N2 Inlet	Outlet
Jun 2000	P	P	n/a	P	P	P	P	P	n/a	P	P	P
Jul 2000	P	P	P	P	P	P	A	P	P	A	P	P
Sep 2000	P	P	P	P	P	P	P	P	P	A	P	A

Table key: P = present enzyme activity; T = trace enzyme activity (limits of assay detection); A = absent enzyme activity; n/a = no sample; RR = rip rap inlet; NE = north east inlet; N1 = north 1 inlet; N2 = north 2 inlet.

Table 4.12 Summary of present/absent/trace of specific enzyme activity for Halbeath pond

Halbeath Pond	Catalase activity				Peroxidase activity				SOD activity			
Month	Inlet	Outlet	North	East	Inlet	Outlet	North	East	Inlet	Outlet	North	East
Jun 2000	P	T	P	P	P	P	P	P	P	P	A	P
3rd Jul 2000	P	A	P	P	P	P	P	P	A	A	A	A
17th Jul 2000	P	P	T	A	P	P	P	P	P	P	P	P
Aug 2000	P	P	P	P	P	P	P	P	P	P	P	P
10th Sep 2000	P	P	P	P	P	P	P	P	P	P	P	P
27th Sep 2000	P	P	T	P	A	T	A	T	P	P	P	P
Jun 2001	P	P	P	P	T	T	T	P	P	P	P	P
Jul 2001	A	A	A	A	A	A	A	A	P	P	P	P
Aug 2001	P	P	P	P	T	T	A	A	P	P	P	P
Sep 2001	n/a	P	n/a	A	n/a	T	n/a	A	n/a	P	n/a	P

Halbeath Pond	Glutathione reductase activity				Glutathione-s-transferase activity			
Month	Inlet	Outlet	North	East	Inlet	Outlet	North	East
Jun 2000	P	P	P	P	P	P	P	P
3rd Jul 2000	P	P	P	P	P	P	P	P
17th Jul 2000	P	P	P	P	P	P	P	P
Aug 2000	P	P	P	P	P	P	P	P
10th Sep 2000	P	P	P	P	P	P	P	P
27th Sep 2000	P	P	P	P	P	P	A	P
Jun 2001	P	P	P	P	T	T	T	T
Jul 2001	P	P	P	P	T	T	T	T
Aug 2001	P	P	P	P	T	T	T	T
Sep 2001	n/a	P	n/a	P	n/a	T	n/a	T

Halbeath Pond	Non-protein SH groups				Total protein SH groups			
Month	Inlet	Outlet	North	East	Inlet	Outlet	North	East
Jun 2000	P	P	P	P	P	P	P	P
3rd Jul 2000	P	P	P	P	P	P	P	P
17th Jul 2000	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Aug 2000	P	P	P	P	P	P	P	P
10th Sep 2000	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
27th Sep 2000	P	P	P	P	P	P	P	P
Jun 2001	P	P	P	P	A	P	A	A
Jul 2001	P	P	P	P	P	P	P	P
Aug 2001	P	P	P	P	T	P	P	T
Sep 2001	n/a	P	n/a	P	n/a	P	n/a	P

Table key: P = present enzyme activity; T = trace enzyme activity (limits of assay detection); A = absent enzyme activity; n/a = no sample.

Table 4.13 Summary of present/absent/trace specific enzyme activity for Pond 7

Pond 7	Catalase activity				Peroxidase activity				SOD activity			
Month	SW Inlet	N Inlet	Outlet	South	SW Inlet	N Inlet	Outlet	South	SW Inlet	N Inlet	Outlet	South
Jul 2000	P	P	P	P	A	A	A	A	A	A	A	P
Sep 2000	P	P	A	A	P	P	P	P	A	A	A	A

Pond 7	Glutathione reductase activity				Glutathione-s-transferase activity			
Month	SW Inlet	N Inlet	Outlet	South	SW Inlet	N Inlet	Outlet	South
Jul 2000	P	P	P	P	A	P	A	P
Sep 2000	P	P	P	P	A	P	P	P

Table key: P = present enzyme activity; T = trace enzyme activity (limits of assay detection); A = absent enzyme activity; n/a = no sample; SW = south west inlet; N = north inlet.

4.4 Discussion

Several researchers have identified increases in the biosynthesis of antioxidants and ROS decomposing enzymes in response to cellular stress (see 4.1). Chapter 3 identified increases in total antioxidant activity in certain *C. glomerata* samples exposed to stress in three urban ponds. This study provides detail as to specific antioxidants where the specific antioxidant profiles of *C. glomerata* will be ascertained and compared with total antioxidant activity, water quality/chlorophyll content and iron/salt stress data. Combining the data obtained from Chapters 2 – 5 may give an insight not only into the health of each pond with respect to pollutant loads, but also improve our physiological and biochemical understanding of the stress response of the common freshwater macroalga *C. glomerata.*

Following the successful of the development of the extraction method and the total antioxidant assay in Chapter 3, the aim of this investigation was to study the specific antioxidant profiles of *C. glomerata* collected from three urban ponds for one year. During year 2 sampling, the project was to focus in more detail on one urban pond Halbeath pond was chosen because it was the oldest pond in the development and was considered to be adequately stable for subsequent studies. Therefore, only algal samples collected from Halbeath pond were assayed for specific antioxidant activities in year two allowing for comparisons to be made in the antioxidant profiles from Halbeath pond throughout a two year period thus giving a greater insight into the pond ecosystem. Furthermore, due to the time consuming nature of measuring sulphydryl content in *C. glomerata* samples, only Halbeath samples from both sampling years were investigated in this study.

4.4.1 Superoxide dismutase activity

Superoxide dismutase was selected for investigation as it directly removes oxygen radicals and is involved in the production of H_2O_2. Elevated levels of H_2O_2 can be found in many stressed organisms, therefore assaying for this enzyme can give an indication of stress in many biological organisms including the alga *C. glomerata* collected from urban ponds. In certain pond samples collected from Linburn pond and Halbeath pond there were relatively high levels of SOD activity with the exception of Pond 7 samples that lacked SOD activity (July south sample contained SOD activity) (Figures 4.1 & 4.2).

Significant increases in SOD activity were detected in Halbeath 2001 samples compared with year 2000 samples in response to oxidative stress ($\chi^2 = 5.636$ with 1 D.F; $p < 0.05$) (Figures 4.2 & 4.15). For both Linburn and Halbeath ponds, the activity of SOD fluctuates between sampling months and in some cases SOD activity was at the limits of detection (Figures 4.1A & 4.2). This indicates that algal antioxidant stability was compromised either due to abiotic factors such as light, temperature and nutrients or low level ROS. Elevated levels of SOD activity, as a result of oxidative stress, have been reported in many algal samples exposed to heavy metals, irradiance, drought and herbicides (Mallick & Mohn, 2000). Despite a lack of significant differences in SOD activity between sampling month and location for both ponds (Linburn and Halbeath pond) (Figures 4.13 & 4.15), the high levels of SOD activity from these algal pond samples are likely to promote an increase in H_2O_2 which may prove toxic to the cells at elevated concentrations and if not removed by catalase/peroxides can lead to the production of the highly toxic hydroxyl radical through Haber-Weiss/Fenton chemistry (see 1.5).

Negligible levels of SOD activity were detected in Pond 7 samples (Figure 4.1B). This may indicate that either SOD is produced in response to normal metabolic processes such as photosynthesis and respiration or it may be exhausted in Pond 7 samples in response to high-levels of stress. SOD produced in response to normal metabolic processes will result in lower levels of H_2O_2 produced by the SOD reaction and may be more readily regulated by catalase/peroxidase activity within the organism. However, if SOD is exhausted then this can result in an accumulation of H_2O_2, which if not removed by catalase/peroxidase may prove toxic to the cells.

4.4.2 Catalase activity

Catalase is located in peroxisomes, but absent from chloroplasts (Van Ginkel & Brown, 1978), and functions to scavenge H_2O_2 generated from the SOD reaction and photorespiration by reducing H_2O_2 to H_2O (Halliwell, 1974; 1982).

Significant differences in catalase activity existed between Pond 7 and Halbeath pond sampling months where increases in activity were detected in Pond 7 July samples ($F_{1,19}$ = 14.85; $p < 0.001$) and Halbeath pond August samples ($H = 13.42$; $p < 0.01$) (Figures 4.3B, 4.4, 4.13 & 4.15). Catalase functions to remove H_2O_2 and therefore increases in catalase activity indicate a response to oxidative stress involving the peroxisomes and an increase in photorespiration. In addition, samples lacking SOD activity or exhaustive SOD activity (Pond 7) were found to contain catalase activity (Figures 4.1B & 4.3B). Again the presence of catalase activity may indicate accumulation of H_2O_2 in the peroxisomes due to photorespiration or H_2O_2 generated from the exhausted SOD reaction. Furthermore, catalase activity was either at the limits of detection or absent in a proportion of algal samples collected from all ponds.

Previous reports have suggested that the alga *Euglena gracilis* lacks the catalase enzyme (Fleck, 2000), but contains a peroxidase enzyme (L-ascorbate peroxidase) to protect cells against peroxides generated during photosynthesis (Brown *et al.*, 1975; Shigeoka *et al.*, 1980). From these studies, it has been shown that *C. glomerata* contains catalase, but due to its low affinity for H_2O_2 in the peroxisomes (Ishikawa *et al.*, 1993), it is likely to contain peroxidase enzymes to regulate intracellular H_2O_2 levels in the chloroplasts and other subcellular compartments.

4.4.3 Peroxidase activity

Peroxidases may be involved in the production of oxidation products with physiological roles and the scavenging of H_2O_2 or organic hydroperoxides (Asada, 1992). The peroxidase assay applied in this study was for guaiacol peroxidases, which produce oxidation products with physiological functions and have been linked to developmental changes (Halliwell, 1982; Amako *et al.*, 1994). Algal samples, which are either absent in peroxidase activity or contain very low detectable levels of activity, may be as a result of the specific nature of guaiacol peroxidase (Figures 4.5 & 4.6). In higher plants, guaiacol peroxidases participate in the biosynthesis of lignin and play important roles in auxin metabolism (Halliwell, 1982; Salin, 1987; Amako *et al.*, 1994). Guaiacol specific peroxidases are therefore not commonly associated with H_2O_2 removal in response to stress induced free radical production. However, significant differences were detected between guaiacol specific peroxidase and sampling month for all three ponds ($H = 19.84$; $p < 0.001$) ($\chi^2 = 20.308$ with 1 D.F; $p < 0.001$) ($H = 9.83$; $p < 0.05$) (Figures 4.5, 4.6, 4.13 & 4.15) and year ($\chi^2 = 36.277$ with 1 D.F; $p < 0.001$) for Halbeath pond (Figures 4.6 & 4.15). Increases in guaiacol specific activity during the specific months and year sampled indicate that the algae may be undergoing developmental changes

rather than oxidative injury. However, it must be noted that samples that do not show guaiacol specific peroxidase may contain other peroxidases such as; ascorbate peroxidase and glutathione peroxidase that function to control H_2O_2 and organic hydroperoxides (Asada, 1992), products of the SOD reaction and lipid peroxidation (Halliwell, 1982). Previous studies have demonstrated that the algae *Euglena gracilis* and *Chlamydomonas reinhardtii* both contain the enzymes ascorbate peroxidase and glutathione peroxidase to metabolise H_2O_2 (Shigeoka *et al.*, 1980; Yokota *et al.*, 1988; Takeda *et al.*, 1992; Overbaugh & Fall, 1985). Therefore, continued studies may benefit from measuring ascorbate peroxidase and glutathione peroxidase in *C. glomerata.*

4.4.4 Glutathione reductase and glutathione-s-transferase activity

The presence of glutathione reductase activity in all samples is likely to function within the chloroplasts, coupled with an ascorbate recycling reaction. Ascorbate peroxidase scavenges H_2O_2 reducing it to H_2O and prevents its involvement in Haber-Weiss/Fenton reactions (Halliwell, 1982). The action of glutathione reductase is in the regeneration of GSH from GSSG.

Relatively high glutathione reductase activity was detected in all algal samples where significant increases in activity were identified in Pond 7 August 2000 samples ($F_{1,18} = 4.53$; $p < 0.05$) and Halbeath pond year 2000 samples ($H = 19.66$; $p < 0.001$) (Figures 4.9B, 4.10, 4.14 & 4.16). Increased glutathione reductase activity indicates exposure to stress where high levels of this enzyme suggests efficient recycling thus helping to protect algal cell membranes from oxidative damage by converting toxic GSSG to GSH. In addition, enhanced levels of glutathione (GSH) as a result of GSSG recycling,

enables GSH to be conjugated with potentially damaging xenobiotics (with GST as the catalyst) (Marrs, 1996) rendering them more water soluble and less toxic to cells (Simons & Vander Jagt, 1977).

Glutathione-s-transferase activity was either at the limits of detection or absent from Linburn pond and Pond 7 and Halbeath 2001 samples. However, GST activity was significantly increased in Halbeath 2000 and east location samples ($H = 12.30$; $p < 0.001$) ($H = 8.06$; $p < 0.05$) concurrent with increases in glutathione reductase activity (Figures 4.10, 4.12 & 4.16). Samples lacking or containing very low detectable levels of GST activity can indicate that they were exposed to excessive xenobiotics stress where the recycled GSH acts a substrate for the xenobiotics effectively rendering it non-toxic to the algae. Samples with increased glutathione reductase and GST activity (Halbeath 2000 samples) (Figure 4.16) suggests that the alga was reacting to oxidative stress. An increase in glutathione reductase activity is likely to be detected as changes in the levels of non-protein bound SH groups, of which GSH is one of the primary classes.

4.4.5 Sulphydryl groups

4.4.5.1 Non-protein bound SH groups

Reduced glutathione (GSH) acts as an important redox buffer, conferring protection by protecting oxygen-sensitive enzymes and susceptible protein thiol groups from oxidative damage by providing a preferential substrate for S-H oxidation (Halliwell, 1982; Packer 1984; Alscher, 1989).

C. glomerata samples collected from Halbeath pond during year 2000 have a significantly higher level of non-protein SH groups compared with year 2001 algal samples ($H = 20.62$; $p < 0.001$) (Figure 4.7, 4.8 & 4.17). The increase in non-protein SH groups are concurrent with increases in glutathione reductase and glutathione-s-transferase (Figures 4.7, 4.10A, 4.12A, 4.16 & 4.17) and indicate that the thiol groups are acting as sites of preferential oxidation but not at the same level as 2001 samples. High glutathione reductase levels coupled with increased non-protein SH groups indicates that enzymatic recycling of GSH from GSSG may be efficient, however, this cannot be confirmed without assaying for these specific enzymes. If this is the case, then GSH may be assumed to be providing protection to oxygen-sensitive enzymes and other proteins from oxidative damage (Halliwell, 1982) in conjunction with neutralizing potentially damaging xenobiotics (Marrs, 1996). However, the continual oxidation of GSH coupled with a reduction in the recycling efficiency of GSH from GSSG may predispose the cells to injury due to the excessive accumulation of GSSG. The increase in non-protein SH groups does suggest that Halbeath pond year 2000 samples were reacting to oxidative stress by increasing the levels of protective GSH.

Non-protein SH levels were low in *C. glomerata* Halbeath 2001 samples compared with Halbeath 2000 samples indicating that preferential oxidation of GSH is occurring within the algal cells where only limited, antioxidant enzyme activity is present in these samples which are capable of regenerating GSH from GSSG. The reduction in non-protein levels is concurrent with decreases in glutathione reductase activity and glutathione-s-transferase activity (Figures 4.8, 4.10B, 4.12B, 4.16 & 4.17) suggesting that the recycling efficiency of GSH from GSSG may be reduced and coupled with the use of limited GSH as a substrate for neutralizing xenobiotics can have a downstream effect on levels of non-protein SH groups.

4.4.5.2 Protein bound SH groups

Halliwell *et al.* (1992) demonstrated that the antioxidants ascorbic acid, protein SH groups, uric acid and α-tocopherol are important agents in protecting cells against nitric oxide (NO_2) and associated peroxidation of lipids. In addition, studies by Hu *et al.* (1993) reported that only protein SH groups from protein amino acid residues were attacked by oxidative damage and may function as an important antioxidant. Therefore, it is likely that protein SH groups may function as preferential sites for oxidation in algal membranes. The elevated level of protein SH groups in Halbeath pond year 2000 *C. glomerata* samples coupled with increased non-protein SH groups indicates that these samples were responding to increased oxidative stress by enhancing the levels of both protein and non-protein bound SH groups (Figure 4.7).

Research carried out by Chevrier *et al.* (1988) demonstrated that ozone (O_3) treatment of *Euglena gracilis* cells caused a decrease in the level of sulphydryl groups which was mainly due to the oxidation of protein SH groups. The findings suggest that protein SH groups may be more accessible to ozone than the SH groups of non-protein compounds. A similar result was also obtained in this study, where Halbeath year 2001 samples contained dramatically reduced protein SH levels and in some cases negative level were obtained compared with non-protein levels, which although reduced in these samples were still present at detectable levels (Figure 4.8). These findings suggest that massive injuries to algal cell membranes occurred due to oxidative damage targeting the membranes. Taking into account the findings by Chevrier *et al.* (1988) with the results obtained in this study, suggests that protein SH groups may be more accessible to oxidative damage than non-protein SH groups.

4.5 Conclusions

From the data obtained it is clear that the *C. glomerata* samples have been exposed to oxidative stress in the ponds and have increased/decreased specific antioxidant activity in response to oxidative stress. However, Halbeath *C. glomerata* year 2001 samples have shown dramatic reductions in their antioxidant enzyme activities indicating that during year 2001 sampling season, the algae may have experienced a massive oxidative stress "assault".

The Halbeath 2001 samples showed decreased glutathione reductase, glutathione-s-transferase and both non-protein and protein SH groups with increased SOD activity (Figure 4.15 - 4.17). Increased SOD activity is likely to cause a significant elevation in H_2O_2 within the algal year 2001 samples, and if the level of H_2O_2 is substantial, it may lead to the depletion of glutathione reductase enzymes and hence non-protein SH groups. In addition, a major "attack" by xenobiotics can cause a further depletion in glutathione-s-transferase and protein SH group levels.

High SOD levels in Linburn pond and Pond 7 algal samples indicate exposure to oxidative stress where elevated SOD may promote increases in H_2O_2, which can be toxic to cells. Negligible SODS levels were detected in certain Pond 7 but catalase activity was present indicating the presence of H_2O_2 in these samples. Relatively high glutathione reductase activity was detected in all samples suggesting efficient recycling of this enzyme, which may help to protect algal cell membranes from oxidative damage.

Chapter 5 will focus on the design of an *in vitro* assay, which will simulate the abiotic stress challenges similar to those found in SUDS ponds using the microalga *E. gracilis.*

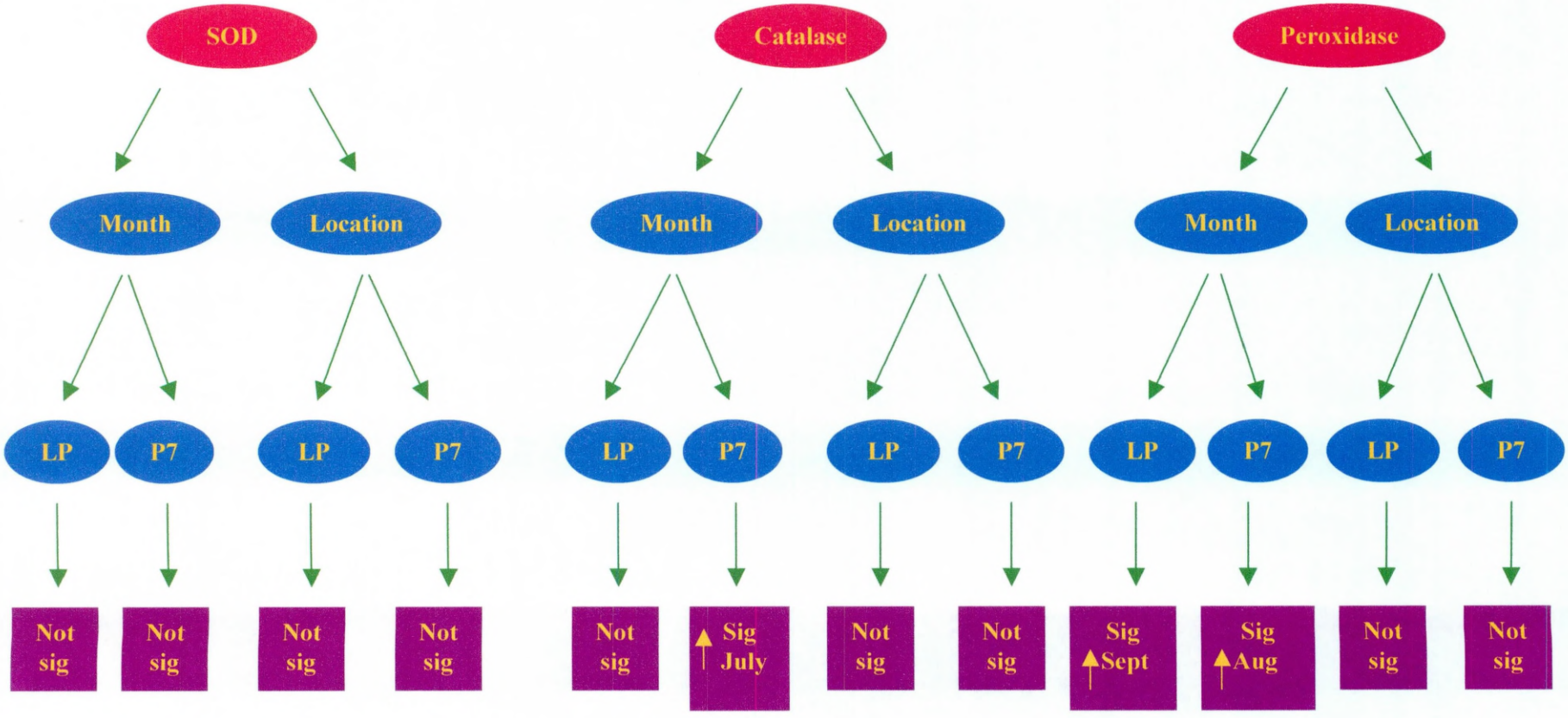


Figure 4.13 Statistical differences in SOD activity, catalase activity and peroxidase activity between sampling month and pond locations for Linburn pond and Pond 7 (Zar, 1996; Sokal & Rohlf, 1981).

Flow key: LP = Linburn pond; P7 = Pond 7; SOD = Superoxide dismutase; Sig = Statistically Significant; Not sig = Not statistically significant.

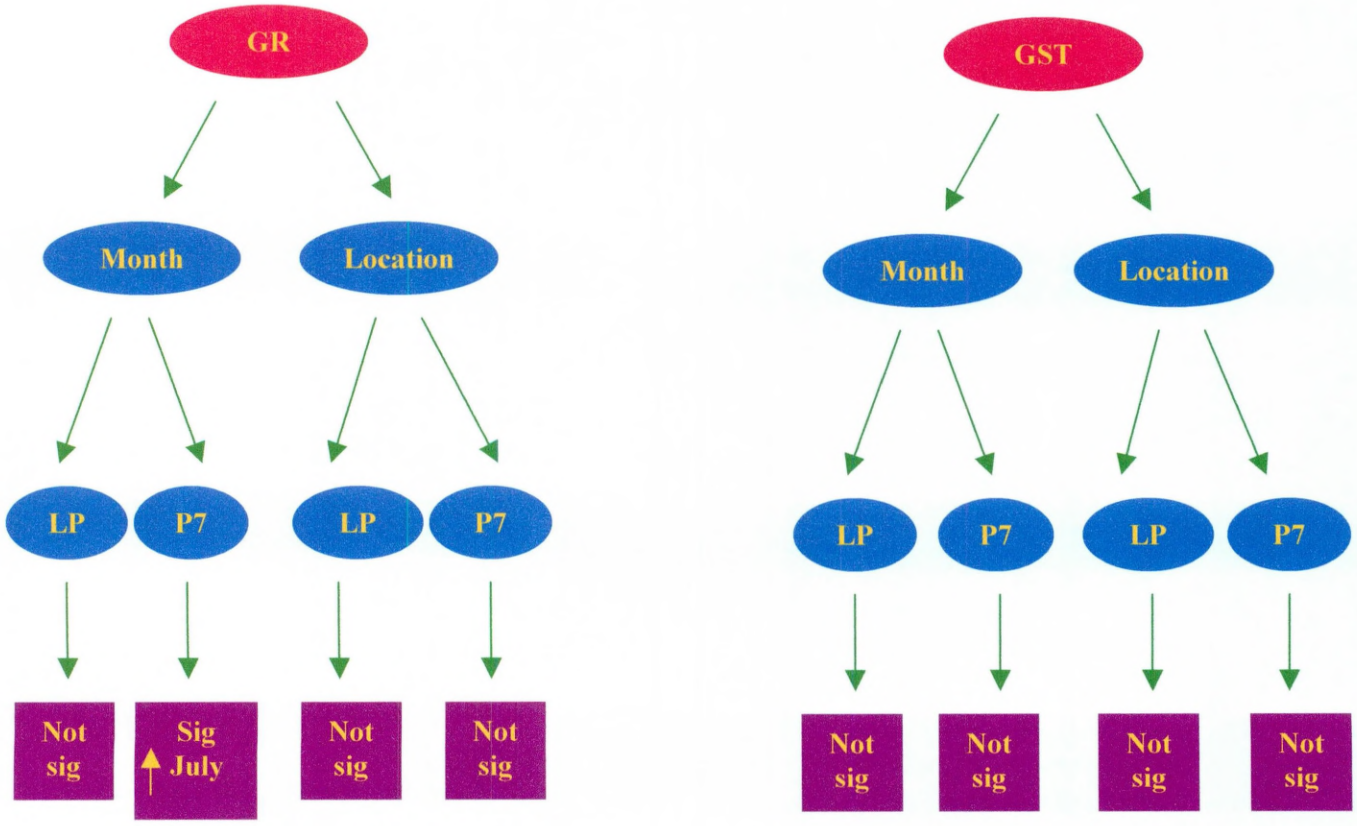


Figure 4.14 Statistical differences in glutathione reductase activity and glutathione-s-transferase activity between sampling month and pond locations for Linburn pond and Pond 7 (Zar, 1996; Sokal & Rohlf, 1981).

Flow key: LP = Linburn pond; P7 = Pond 7; GR = Glutathione reductase; GST = Glutathione-s-transferase; Sig = Statistically Significant; Not sig = Not statistically significant.

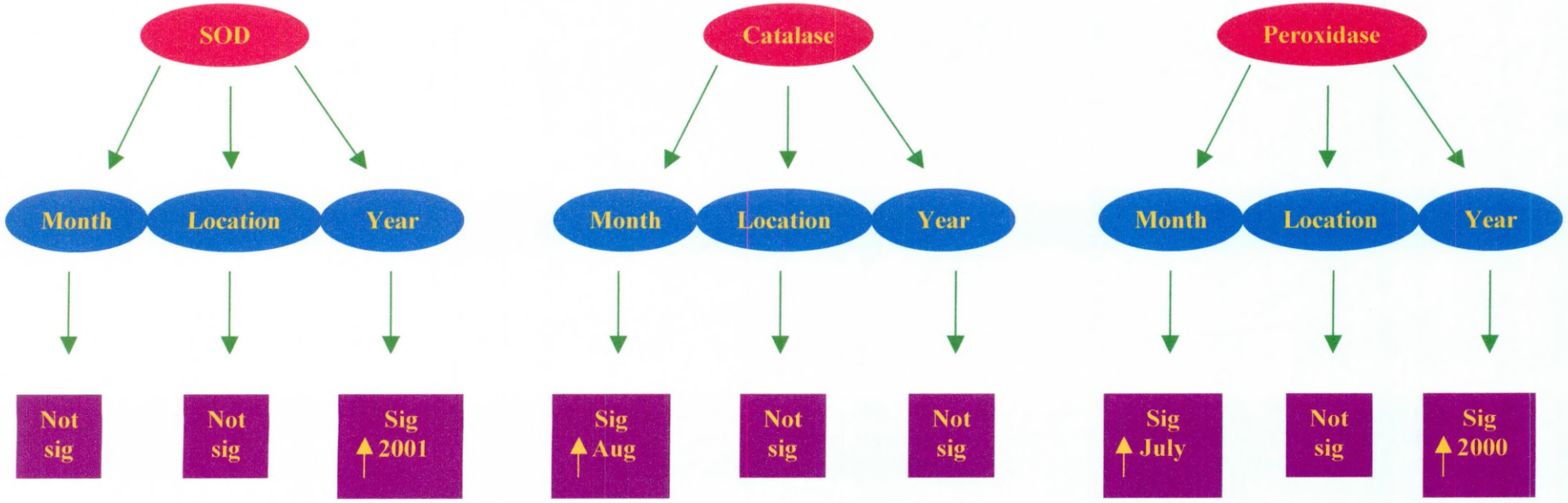


Figure 4.15 Statistical differences in SOD activity, catalase activity and peroxidase activity between sampling month, pond location and sampling year for Halbeath pond (Zar, 1996; Sokal & Rohlf, 1981).

Flow key: SOD = Superoxide dismutase; Sig = Statistically Significant; Not sig = Not statistically significant.

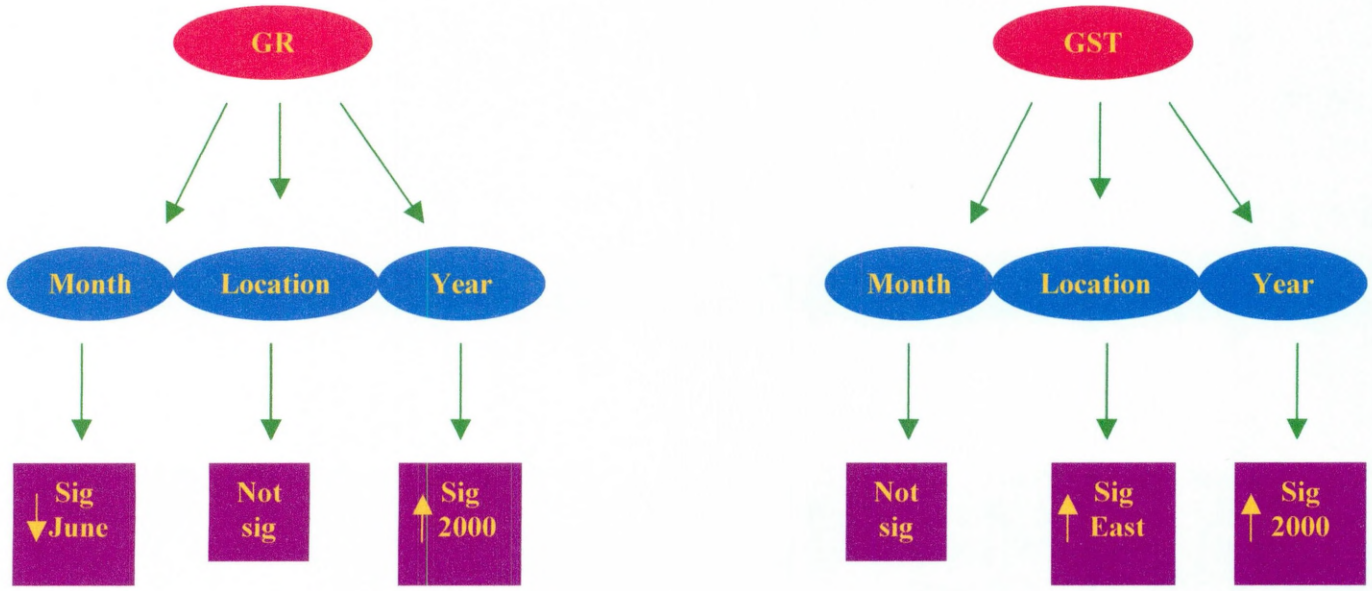


Figure 4.16 Statistical differences in glutathione reductase activity and glutathione-s-transferase activity between sampling month, pond location and sampling year for Halbeath pond (Zar, 1996; Sokal & Rohlf, 1981).

Flow key: GR = Glutathione reductase; GST = Glutathione-s-transferase; Sig = Statistically Significant; Not sig = Not statistically significant.

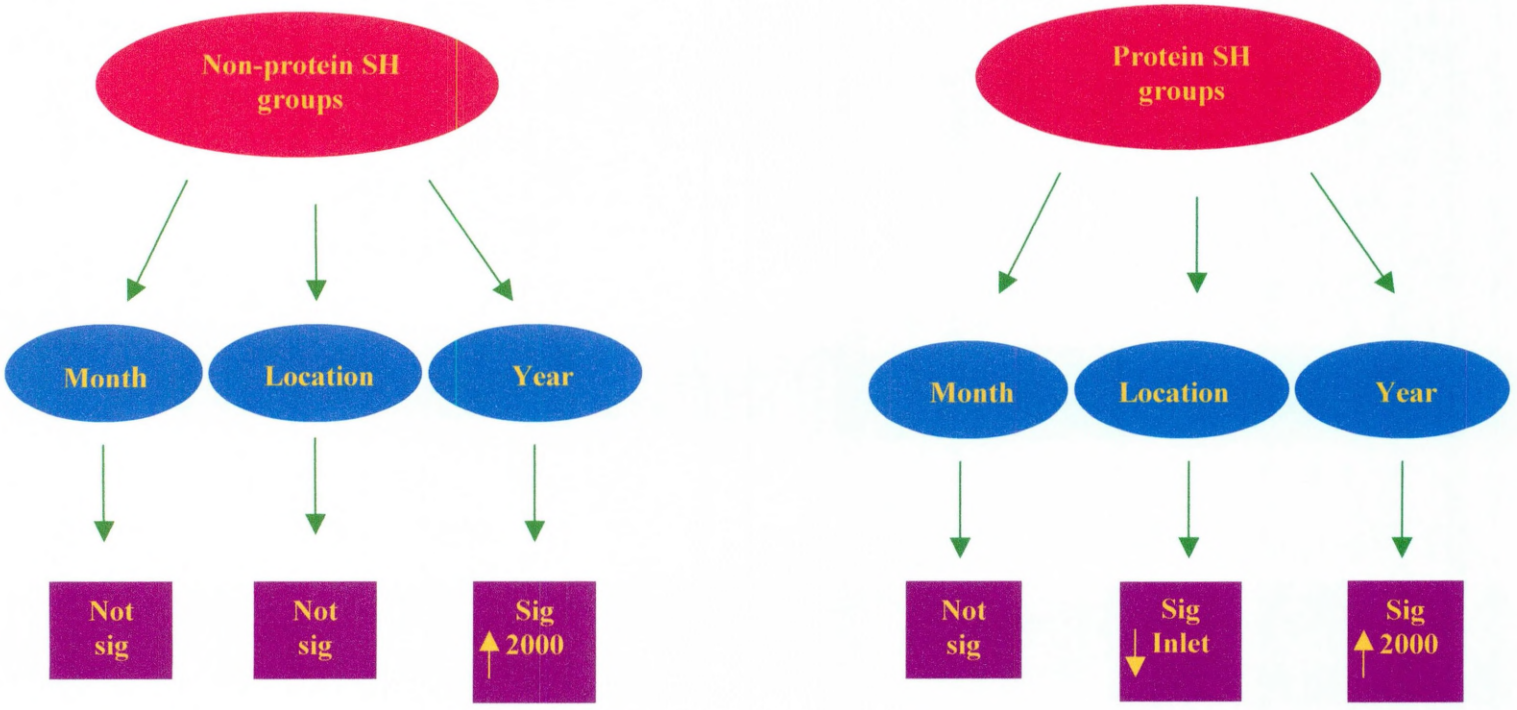


Figure 4.17 Statistical differences in non-protein and protein SH groups between sampling month, location and year for Halbeath pond (Zar, 1996; Sokal & Rohlf, 1981).

Flow key: Non-protein SH groups = Non-protein sulphydryl groups; Protein SH groups = Protein sulphydryl groups; Sig = Statistically Significant; Not sig = Not statistically significant.

Chapter 5 DEVELOPMENT OF AN *IN VITRO* TEST TO STUDY ALGAL RESPONSES TO ABIOTIC STRESS IN URBAN WATER

5 Introduction

Exposure of algae to many biotic and abiotic stresses including; temperature extremes, heavy metals, herbicides and radiation can induce the production of oxidative markers. This can also be used as a generic stress response in algae (Rady *et al.*, 1994; Okamoto & Colepicolo, 1998; Vartak & Bhargava, 1999). The development of an *in vitro* assay to detect oxidative markers in the microalga *Euglena gracilis* can thus provide a useful tool in an aquatic monitoring programme and also highlights the importance of algae as a pollutant indicator. In addition, *E. gracilis* may have the potential to be further utilised in clean-up programmes of wastewater using the process of phytoremediation.

Water quality and sediment data collected from each pond during the three-year study showed that the ponds contain relatively elevated chloride and iron concentrations. Higher chloride levels, especially during the winter, and early spring months are due to salt applications on road surfaces close to the pond sites (2.4.3 and Figure 2.10) and high iron deposits in the pond sediments from iron ore in the surrounding area (Appendix 1). In the context of this study, the objective of this chapter was to design an *in vitro* assay to simulate abiotic stress challenges similar to those found in SUDS ponds. Cultures of the microalga *E. gracilis* were exposed to high saline (NaCl) and iron ($FeSO_4$) concentrations *in vitro,* within ranges detected in SUDS ponds. Furthermore, simulating the standard units used to report the analysis of the compounds in the ponds using SEPA recommended levels ensures that the information can be

correlated directly to SEPA environmental standards. Therefore, exposing cultures of *E. gracilis* to abiotic stress (NaCl and $FeSO_4$) may induce oxidative markers and hydroxyl radicals within the alga, which can then be monitored using the total antioxidant assay, volatile sampling and gas chromatography. It was hypothesised that by simulating the xenobiotic conditions found in the ponds, *in vitro,* (using the test organism *E. gracilis*), an insight into the tolerance/sensitivity of the algae will be given. In addition, it may also be possible to determine whether these concentrations elicit a stress response *in vitro* in *E. gracilis* and hence by extrapolation if this indicates a potential response *in vivo* (urban ponds) by *C. glomerata* and other algal species. The assays may therefore be used to test the effects of urban waters from SUDS on a biotic basis. Furthermore, the assays and testing approach may be used to screen for potential phytoremediators from algae distributed in SUDS.

5.1 The test organism *E. gracilis*

C. glomerata proved problematic for use in this assay due to difficulties with standardization of inoculum, growth measurement and obtaining adequate replication in relation to algal sensitivity to heavy metal pollution. As a result, *C. glomerata* was deemed an unsuitable candidate as a test organism and was replaced with the unicellular euglenoid *E. gracilis*. This alga was chosen due to its ease of culturing, standardisation of inoculum, ease of replication and its wide distribution in many aquatic systems (Appendix 2B). In addition, work carried out by Fleck (1998) and Fleck *et al.*, (2000), using this organism, had successfully demonstrated detection of oxidative markers using gas chromatography monitoring. Thus this study aimed to establish if *E. gracilis* is a useful *in vitro* indicator of pond health and it may also offer the possibility of a potential use in phytoremediation programmes for the enhanced quality of wastewater effluent.

5.1.1 Free radical markers

In certain conditions oxygen, the primary agent of aerobic respiration and metabolism, has the potential to be one of the most important promoters of cellular damage in biological systems as it can lead to the formation of highly reactive and destructive oxyradicals (Benson, 1990). One of the most damaging activated oxygen species is hydrogen peroxide as it can easily penetrate cell membranes and also propagate oxidative damage by reacting with superoxide radicals (catalysed by transition metal ions) to produce the most oxidising and damaging of all reactive oxygen species (ROS), the hydroxyl radical (Benson 1990). In this study, it was hypothesised that by exposing *E. gracilis* to excess iron the alga will not only produce hydrogen peroxide and superoxide radicals in response to iron stress, but it will also have a surplus supply of Ferrous ions to further enhance hydroxyl radical levels by participating in the Fenton reaction (Figure 5.1). The hydroxyl radical can therefore act as an ROS marker of stress and be detected using gas chromatography by using dimethyl sulphoxide as a $^{\cdot}OH$ probe, which produces methane in headspace samples.

$$H_2O_2 + O_2^{\cdot-} \xrightarrow{Fe^{2+}/Fe^{3+}} {}^{\cdot}OH + {}^{-}OH + O_2$$

Figure 5.1 The Fenton reaction (Halliwell & Gutteridge, 1989).

5.1.2 Monitoring of free radicals

Due to their highly reactive state the only direct means by which free radicals can be monitored is using Electron Paramagnetic Resonance (EPR), usually in combination with spin traps (Benson & Bremner, 2002). Although EPR spectroscopy allows both quantification and characterization of free radicals, under physiological conditions, only a small number of free radicals in biological systems are sufficiently stable to be

detected by EPR (Doorslaer *et al.*, 1999). Alternatively, indirect detection using "marker" compounds that are formed as a result of free radical reactions can be applied. In this study, indirect detection of hydroxyl radicals were used as the ROS marker due to work carried out by Benson & Withers (1987) and Fleck (1998; 2000) who successfully studied oxidative stress in *Daucus carota* and *E. gracilis* cells respectively, undergoing cryopreservation related stress and cryoinjury. Hydroxyl radicals, an ROS marker, can be trapped with a molecular probe to yield detectable products that correlate to stress impacts in a range of organisms. There are many molecular probes such as benzoic acid, methional, 2-keto-4-thiomethylbutanoic acid (KTBA) and dimethyl sulfoxide (DMSO) all of which detect hydroxyl radicals in simple reaction systems that contain minimum concentrations of competing $^{\cdot}OH$ scavengers (Babbs & Steiner, 1990). DMSO was therefore utilised in this study as a hydroxyl radical probe, where methane (the stable volatile reaction product of $^{\cdot}OH$ and DMSO) was used as a marker for $^{\cdot}OH$ using headspace volatile sampling and gas chromatography (Figure 5.2).

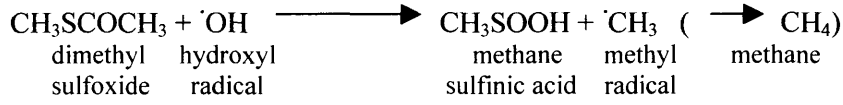


Figure 5.2 Methane production from DMSO (Benson & Bremner, 2003)

Methane detected by gas chromatography is related to hydroxyl radical production, therefore it is hypothesised that algal samples exposed to excess salt and iron, emit methane in the presence of DMSO and thus exhibit a stress response.

5.1.3 Antioxidant markers

Biological systems have developed diverse and highly complex defences against free radical attack, they function to destroy the radicals and toxic oxygen species as soon as

they are formed. Antioxidant defences consist of both enzymatic and non-enzymatic mechanisms (Karpinski *et al.*, 2000). Enzymatic antioxidants confer protection by scavenging the radical species thus removing ROS and reducing cellular damage. In this study, the total antioxidant assay developed in Chapter 3, was used in combination with gas chromatography to determine the extent of free radical scavenging by *E. gracilis* antioxidant enzymes and this gives an indication of stress levels in the organism.

5.1.4 Viability markers

The viability of *E. gracilis* post experiment (120hr) was assessed using the vital stain Fluorescein Diacetate (FDA). FDA was chosen due to the successful use of applying this stain to identify a selection of viable Antarctic micro and macroalgae (Johnstone *et al.*, 2002). In the context of this chapter, viability assessments of the alga post experiment can give an immediate indication of algal health and therefore provides a useful marker "tool" for detecting those samples with which exposure to iron and salt has had the most detrimental effect.

5.2 Materials and Methods

5.2.1 Organism and culture regimes

The freshwater eukaryotic microalga *E. gracilis* Klebs CCAP 1224/5z was selected for study from the Culture Collection of Algae and Protozoa (CCAP) (Tompkins *et al.*, 1995). Culture medium was prepared in advance and from stock solutions to ensure consistent quality and ease of production. Stock solutions of EG, JM and EG:JM was prepared in deionised water (dH_2O) and refrigerated at 4°C, with the exception of JM

stock solution 9 which was stored at room temperature (Table 6.1). Stock solutions were added to a large proportion of the final volume of media and then made up to the volume with dH_2O. The self-buffering media was dispensed into culture vessels (150ml conical flasks) and then autoclaved at 121°C (100 kPa) for 15min.

E. gracilis cultures were maintained at 15°C under a 12:12hr light:dark regime. Illumination was provided by cool white fluorescent lamps with a photon flux density of 50μmol.m^2 s^{-1} at the surface of the culture vessel.

Sub-culturing of *E. gracilis* was performed using standard microbiological techniques. A 1ml inoculum of *E. gracilis* was asceptically transferred from a late log/stationary phase culture into fresh sterilised EG:JM medium (80ml). Transfer intervals for *E. gracilis* maintained under standard culture conditions varied between 4 and 5 weeks.

Table 5.1 Jaworski's medium (JM)

Stock No.	Compounds	Per 100 ml
1	$Ca(NO_3)_2.4\ H_20$	2.0g
2	K_2HPO_4	1.44g
3	$MgSO_4.7\ H_20$	5.0g
4	$NaHCO_3$	1.59g
5	EDTA (FeNa Salt)	0.225g
	EDTA (Na_2 Salt)	0.225g
6	H_3BO_3	0.248g
	$MnCl_2.4\ H_20$	0.138g
	$(Na_4)_6Mo_7O_{24}.4\ H_20$	0.10g
7	Cyanocobalamin	0.004g
	Thiamine HCl	0.004g
	Biotin	0.004g
8	$NaNO_3$	8.0g
9*	$Na_2HPO_4.12\ H_20$	3.6g

(*stored at room temperature)

JM was prepared by the addition of 1ml of each stock solution to 1 litre dH_2O (Tompkins *et al.*, 1995).

E. gracilis medium (EG)

EG was prepared by the addition of 10mls $CaCl_2$ stock solution ($1g.litre^{-1}$), Sodium acetate trihydrate (1.0g) (Sigma), 'Lab-Lemco' powder (1.0g), Tryptone (2.0g), and Yeast extract (2.0g) to 1 litre dH_2O (Tompkins *et al.*, 1995). Chemicals were obtained from Oxoid Laboratories unless otherwise stated.

EG:JM medium

EG:JM medium was prepared using a 1:1 mixture of EG and JM which was mixed then autoclaved at 121°C (100 kPa) for 15min (Tompkins *et al.*, 1995).

5.2.2 Viability assessment using fluorescein diacetate vital stain

E. gracilis cells were examined using a Leitz Labourlux microscope equipped for bright field and fluorescence microscopy. Cells were mounted on microscope slides over which a coverslip was gently lowered. *E. gracilis* viability was assessed using fluorescence microscopical assessment with the vital stain Fluorescein Diacetate (FDA) (Sigma). Viable *E. gracilis* cells with functioning esterase cleave the stain that then fluoresces an intense yellow/green under UV illumination. Non-viable cells can either appear colourless or red due to autofluorescence of chlorophyll.

A 0.1% (w/v) stock solution of FDA in 100% acetone was filter sterilised (0.2μm filter) and stored at 4°C. Prior to use, the stain was diluted to $1\mu l.ml^{-1}$ of EG:JM culture medium. A few drops of the diluted stain were added to *E. gracilis* cells on a microscope slide and observed under UV illumination (Harding & Benson, 1995). Viability was assessed as the percentage of cells that fluoresced as compared to the total number of cells that did not fluoresce, by comparing dark and light fields of view.

Cell counts were performed on an Improved Neubauer haemocytometer. *E. gracilis* suspensions were transferred to both chambers of the haemocytometer using a Pasteur pipette by touching the edge of the cover slip with the pipette and filling each chamber by capillary action. For each chamber, cells within the 0.04 mm^2 central and four corner squares of the same dimensions were counted.

Each square of the haemocytometer with the coverslip represents a total volume of 0.004 mm^3 or $4x10^{-6}$ cm^3.

Cells per ml = average count per 0.004 mm^2 x dilution factor x 250,000.

5.2.3 Volatile hydrocarbon analysis by gas chromatography

5.2.3.1 Vial preparation

Glass vials with an accurately determined volume of 4.65 ml $\pm$ 0.02ml were used throughout this study. Vials were loosely sealed using airtight silicon-teflon septa (Suplico) and sterilised by autoclaving at 121°C/10bar. Immediately after sterilisation, the vials were transferred to a laminar flow cabinet, opened and vented for 30min to remove unwanted volatile build-up due to autoclaving.

5.2.3.2 Preparation of *E. gracilis* for gas chromatography

E. gracilis cell suspensions were cultured under standard conditions (5.2.1). Immediately prior to the addition of *E. gracilis* to the vials, cells from a single culture were removed under aseptic conditions and a cell count performed using the method as described in section 5.2.2. *E. gracilis* cultures of 10^6 cells/ml were used throughout this experiment. Cell suspensions and appropriate controls (Table 5.2) were placed in pre-

prepared vials (4ml) and the vials sealed with airtight silicon-teflon septa. The time at which the vials were sealed was noted to permit calculation of the duration between sealing and injecting the headspace samples into the gas chromatography machine allowing methane production.hr to be determined. Vial headspace was analysed by gas chromatography for volatile hydrocarbons at intervals of 24hr, 48hr, 72hr, 96hr and 120hr respectively, with venting of the vials following sampling.

Table 5.2 GC vial constituents

Sample	Constituents	Volume (µl)
Control 1	1% (w/v) DMSO*; EG:JM media; NaCl (5000ppm)/ $FeSO_4$ (10% w/v)*	20; 20; 1960
Control 2	*E. gracilis*; EG:JM media; NaCl (5000ppm)/ $FeSO_4$ (10% w/v)*	1960; 20; 20
Control 3	*E.gracilis*; 1% (w/v) DMSO*; EG:JM media	1960; 20; 20
Sample 1	*E.gracilis*; 1% (w/v) DMSO*; NaCl (10ppm)*	1960; 20; 20
Sample 2	*E.gracilis*; 1% (w/v) DMSO*; NaCl (100ppm)*	1960; 20; 20
Sample 3	*E.gracilis*; 1% (w/v) DMSO*; NaCl (500ppm)*	1960; 20; 20
Sample 4	*E.gracilis*; 1% (w/v) DMSO*; NaCl (1000ppm)*	1960; 20; 20
Sample 5	*E.gracilis*; 1% (w/v) DMSO*; NaCl (5000ppm)*	1960; 20; 20
Sample 6	*E.gracilis*; 1% (w/v) DMSO*; $FeSO_4$ (2% w/v)*	1960; 20; 20
Sample 7	*E.gracilis*; 1% (w/v) DMSO*; $FeSO_4$ (4% w/v)*	1960; 20; 20
Sample 8	*E.gracilis*; 1% (w/v) DMSO*; $FeSO_4$ (6% w/v)*	1960; 20; 20
Sample 9	*E.gracilis*; 1% (w/v) DMSO*; $FeSO_4$ (8% w/v)*	1960; 20; 20
Sample 10	*E.gracilis*; 1% (w/v) DMSO*; $FeSO_4$ (10% w/v)*	1960; 20; 20

*DMSO, NaCl and $FeSO_4$ were pre-prepared in dH_2O and filter sterilised before use.

5.2.3.3 Gas chromatography

Headspace samples (1ml) were drawn from the vials and injected into a Perkin-Elmer 8310 Gas Chromatograph (Perkin-Elmer, USA) fitted with a 2m Poropak Q column (Supelco, UK) and a hydrogen/air flame ionisation detector. Volatiles were separated using a temperature programme with an oven temperature of 35°C and an isothermal time of 6min. The injector and detector temperatures were 200°C and 225°C

respectively. Nitrogen was used as the carrier gas at a flow rate of 20ml.min^{-1}. The volatile hydrocarbon chromatographic peak of methane was identified using a standard 15ppm gas mixture. At all stages, appropriate controls were incorporated into the analyses to determine the presence of background volatiles emitted from DMSO, media, NaCl, $FeSO_4$ and the laboratory atmosphere. Where necessary background levels of methane were subtracted from the *E. gracilis* sample data (Benson & Withers, 1987).

Headspace samples (1ml) were drawn from the vials using a 5ml gas-tight syringe. In order to ensure complete mixing of the headspace, the syringe was flushed several times before removal. After sampling, the vials were aerated in a laminar flow bench for 30min, re-sealed (time noted) and returned to the growth cabinet.

Methane production.hr was calculated using data corrected for background methane levels. Data was expressed as parts per million (ppm) methane (as designated by the standard gas mixture) per 10^6 *E. gracilis* cells.

5.2.4 *E. gracilis* extraction for antioxidant analysis

Following completion of GC analyses (120hr) and viability assessment, the *E. gracilis* cell suspensions were extracted using a modified version of the extraction procedure as detailed in 3.2.2.2 – 3.2.2.5. Cell suspensions (1ml) were removed from the sample and control vials and centrifuged (Jouan A14) at 5000rpm for 30 sec. The supernatant was removed, 1ml chilled (on ice) extraction buffer (3.2.2.1) was added and the suspension sonicated (on ice) continuously for 2 min at 30% amplitude (600 Cole Parmer 4 mm microtip). To remove large algal aggregates, the cell suspension was filtered through a Grade 1 Whatman filter with the aid of a vacuum pump (Neuberger-N86 KN18), centrifuged in a cold room (5°C) using a pre-cooled centrifuge (5°C) (Jouan A14) at

14,000rpm for 10min. The supernatant was transferred to a fresh Eppendorf (2ml) and stored at –20°C until required.

5.2.5 Total antioxidant assay

To recover cell suspensions for assaying, the extracts were removed from the –20°C freezer, thawed and maintained on ice. Total antioxidant status of the cell extracts was determined using the method detailed in 3.2.4.

5.2.6 Statistical analysis

Statistical analysis of methane and viability results from *E. gracilis* was not applied due to a lack of methane gas emitted from the samples and consistency of sample variation between replicates for algal viability. A tally for discrete variables using Minitab v.13 (Minitab, USA) identified only 3 from a possible 150 samples (salt study) that contained methane above background levels, and no methane was detected above background levels for algal samples containing iron. Viability of all algal samples (both salt and iron studies) > 90% with little variation between replicate samples therefore it was not appropriate to analyse this data using Minitab.

Total antioxidant activity results were analysed separately for both salt and iron studies using a one way analysis of variance (ANOVA). Comparisons of samples with the controls were investigated using Dunnett's test with a 5% error rate (Zar, 1996).

5.3 Results

5.3.1 Gas chromatography

Despite the lack of methane generated by many of the algal samples, methane was detected by gas chromatography in selected control and samples tubes and thus proved to be an effective and accurate method for assaying volatile hydroxyl radical markers and DMSO, an efficient scavenger of hydroxyl radicals.

5.3.2 Methane production

5.3.2.1 Salt stress

Methane production was above background levels in *E. gracilis* cells treated with 10ppm NaCl, detected at 72hr (0.042ppm.CH_4.hr.10^6 cells) and 1000ppm NaCl detected at 120hr (0.003 ppm.CH_4.hr.10^6 cells) (Table 5.3).

5.3.2.2 Iron stress

Control 1 *E. gracilis* iron samples were the only samples to produce methane at concentrations of 0.033($\pm$ 0.02), 0.053($\pm$ 0.012), 0.042($\pm$ 0.039) and 0.023($\pm$ 0.028) ppm.CH_4.hr.10^6 (cells), during the period of 24, 48, 72 and 120hr respectively.

Table 5.3 Time course of the effect of NaCl on CH_4 production by *E. gracilis*

NaCl	ppm.CH_4.hr.10^6 (cells)				
Sample	**24**	**48**	**72**	**96**	**120**
Control 1	0.049 (±0.067)	0.003 (±0.007)	0	0.019 ±0.031)	0.010 (±0.014)
Control 2	0.034 (±0.077)	0.007 (±0.016)	0	0	0
Control 3	0.006 (+0.012)	0.019 (±0.022)	0	0.001 (±0.001)	0
10 ppm	0	0	0.042 (±0.073)	0	0
100 ppm	0	0	0	0	0
500 ppm	0	0	0	0	0
1000 ppm	0	0	0	0	0.003 (±0.009)
5000 ppm	0	0	0	0	0

NaCl – CH_4 production.hr.10^6 cells between control samples and *E. gracilis* samples containing 10–5000 ppm NaCl.

[Control 1 - 1% (w/v) DMSO; EG:JM media; NaCl (5000ppm)/$FeSO_4$ (10% w/v); Control 2 - *E. gracilis*; EG:JM media; NaCl (5000ppm)/$FeSO_4$ (10% w/v) and Control 3 - *E. gracilis*; 1% (w/v) DMSO; EG:JM media]

n = 5, error terms are expressed as standard deviations.

5.3.3 Viability assessment

Figure 5.3 illustrates that viability levels for all control and *E. gracilis* samples pre-treated with either NaCl or $FeSO_4$ were > 90% viability with little or no variation between replicate samples (error bars are not applicable for these samples).

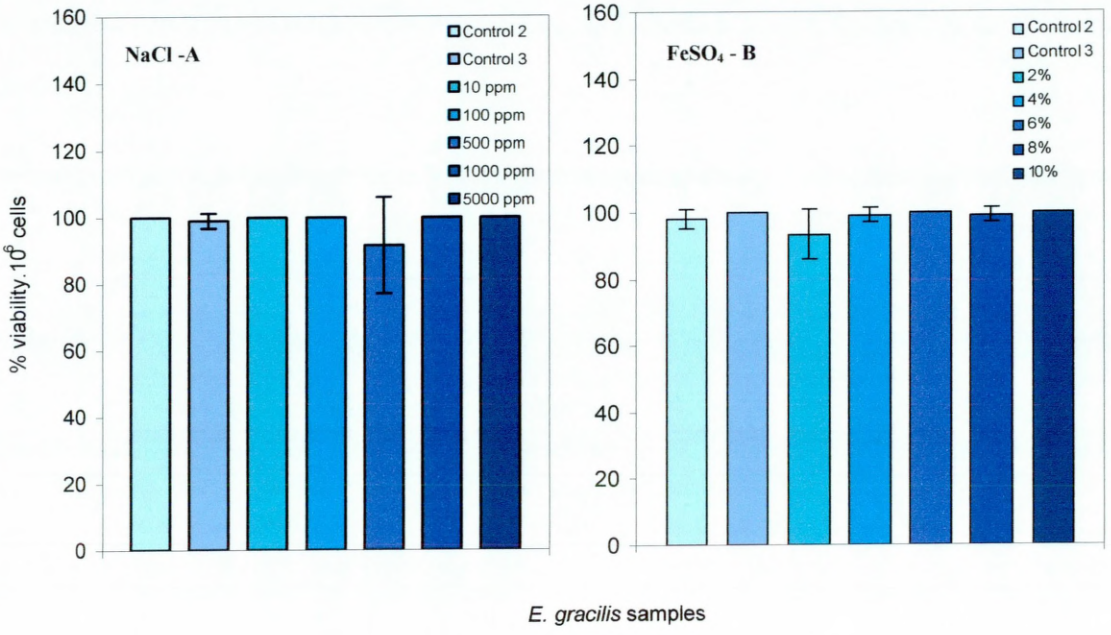


Figure 5.3 The effects of NaCl and $FeSO_4$ on post-treatment viability in *E. gracilis*, assessed by FDA staining (at day 5 of experiment).

A and B - % viability in *E. gracilis* control and treatment samples analysed using FDA stain during day 5 of experiment.

[Control 2 - *E. gracilis*; EG:JM media; NaCl (5000ppm)/$FeSO_4$ (10% w/v) and Control 3 - *E. gracilis*; 1% (w/v) DMSO; EG:JM media]

n = 5, error terms are expressed as standard deviations.

5.3.4 Total antioxidant activity

5.3.4.1 Salt stress

Significant differences were detected in the total antioxidant activities between all *E. gracilis* samples pre-treated with 10 – 5000ppm NaCl compared with control 2 activities when analysed using a one way ANOVA with Dunnett's comparisons ($F_{5,14}$ = 57.15; $p < 0.001$) (Figure 5.4 A).

Analysis of *E. gracilis* total antioxidant activities using a one way ANOVA with Dunnett's comparison illustrates that samples pre-treated with 10ppm NaCl show no significant difference compared with control 3 activities. Significant differences were detected in the antioxidant profiles of *E. gracilis* samples pre-treated with 100 – 5000ppm NaCl compared with control 3 samples ($F_{5,14}$ = 22.18; $p < 0.001$) (Figure 5.4 A).

5.3.4.2 Iron stress

Significant differences were detected in the total antioxidant activities between *E. gracilis* samples pre-treated with 2%, 4%, 8% and 10% (w/v) $FeSO_4$ compared with control 2 and control 3 activities ($F_{5,29}$ = 38.82; $p < 0.001$) ($F_{5,29}$ = 48.88; $p < 0.001$) (Figure 5.4 B). However, no significant difference was detected in *E. gracilis* cells pre-treated with 6% (w/v) $FeSO_4$ when compared with control 2 and control 3 antioxidant activities (Figure 5.4 B).

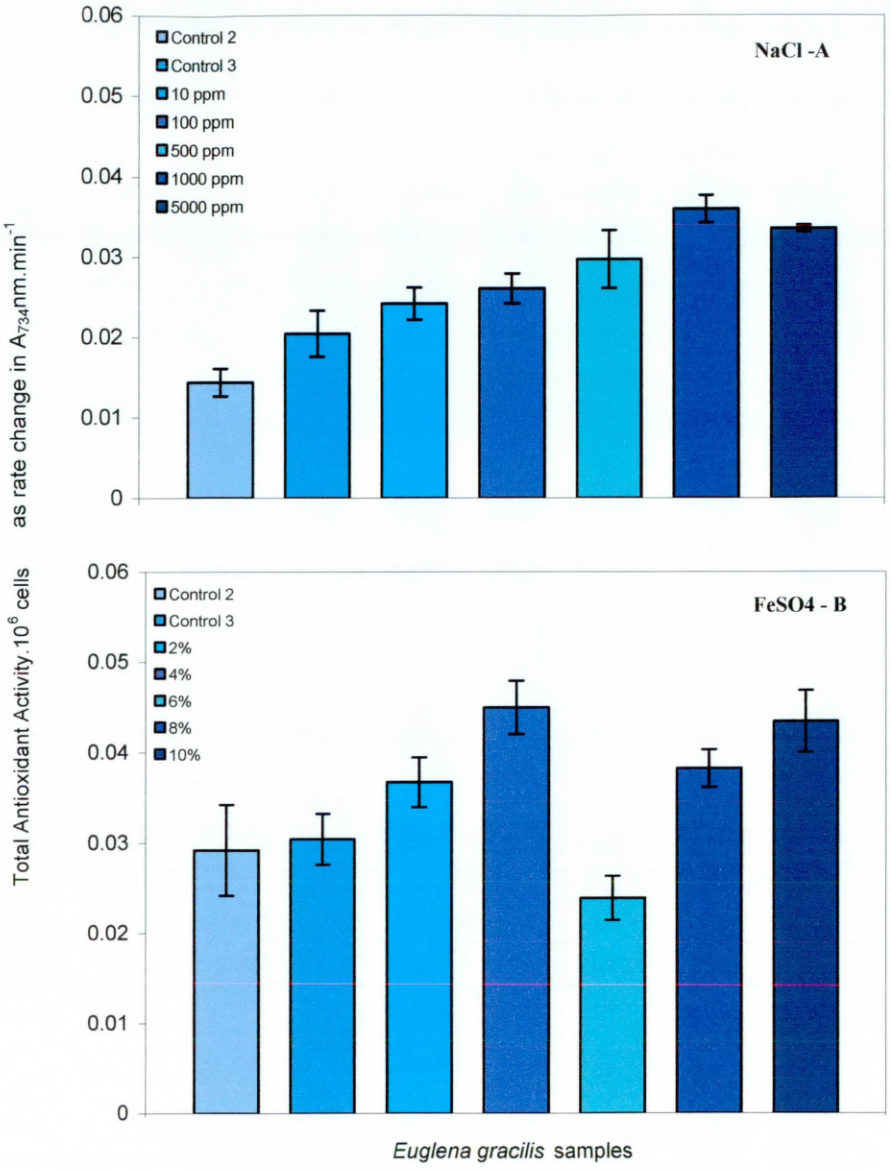


Figure 5.4 The effects of NaCl and $FeSO_4$ on total antioxidant status in *E. gracilis,* assessed using the total antioxidant assay (at day 5 of experiment).

A – Profile of total antioxidant activity in *E. gracilis* exposed to various NaCl concentrations with controls and B - profile of total antioxidant activity in *E. gracilis* exposed to various $FeSO_4$ concentrations with controls.

[Control 2 - *E. gracilis*; EG:JM media; NaCl (5000ppm)/$FeSO_4$ (10% w/v) and Control 3 - *E. gracilis*; 1% (w/v) DMSO; EG:JM media]

n = 5, error terms are expressed as standard deviations.

5.4 Discussion

The objective of this chapter was to design an *in vitro* assay to stimulate abiotic stress challenges similar to those found in SUDS ponds using *E. gracilis* as a test organism. The assay is based on assessing a generic stress response in algae, and aims to give an insight into the tolerance/sensitivity of the alga to high saline and iron concentrations. It also highlights the potential importance of the algae as a pollutant indicator in aquatic monitoring programmes. In this respect, it may be used in testing collected water samples. Furthermore, data presented in this chapter indicate that this organism may also be further utilised in clean-up programmes of wastewater using the process of phytoremediation to enhance effluent quality.

5.4.1 Methane production

Fleck *et al.* (2000) used the DMSO/methane evolving ·OH radical probe system to successfully identify the components of a cryopreservation protocol that were most damaging to the microalga *E. gracilis.* The same protocol was applied in this study due to its non-destructive nature and the fact that sequences of different treatments can be examined as cumulative stress parameters. However, a major disadvantage with this system is that the hydroxyl radicals, due to their high reactivity, can readily oxidize proteins, nucleic acids and purines, all of which are found in biological systems. Hydroxyl radicals are much more likely to react with various endogenous biological compounds in these systems rather than with the introduced molecular probe (Babbs & Steiner, 1990). Despite these disadvantages, DMSO is a highly penetrating solvent and is likely to enter *E. gracilis* cells effectively as was demonstrated by Fleck *et al.* (2000). It is most likely that if ·OH were present in the samples in response to iron/salt stress then methane would have been detected. Therefore, the lack of methane detected in

stressed *E. gracilis* samples indicates that antioxidants were preventing the formation of ·OH radicals before they reacted with DMSO to emit methane. However, two *E. gracilis* samples exposed to 10ppm and 1000ppm NaCl did emit methane at 72hr and 120hr respectively (Table 5.3). It is hypothesised that at 72hr, the level of antioxidants within the alga may have become saturated thus resulting in less ·OH radical scavenging and an increase in methane levels. However by 96hr the level of antioxidant production may have increased sufficiently to scavenge ·OH radicals and thus no methane was detected in either 96hr or 120hr samples. *E. gracilis* samples exposed to 1000ppm NaCl at 120hr may not only be experiencing senescence but also increased stress as a result of prolonged exposure to NaCl. Both of these factors can result in a decrease in antioxidant activity and uncoupling of the antioxidant system thus resulting in less ·OH radical scavenging and increased methane levels. Control samples (1) from both studies (these lack *E. gracilis* cells) produced methane above background levels and supports the hypothesis that samples containing algae were not generating CH_4 due to antioxidant scavenging of ·OH radicals. Small quantities of methane were also detected above background levels in control samples 2 and 3 from the salinity investigation, but not from the same controls in the iron study (Table 5.3). Methane derived from these controls is most likely evolved from the medium, septa, vessels or the laboratory atmosphere and the lack of methane derived from the same controls in the iron study supports this theory.

5.4.2 Viability

Viability in *E. gracilis* cells was monitored with a view to correlating recovery with free radical activity and antioxidant status as evidenced by methane evolution. Results from Figure 5.3 show that neither NaCl nor $FeSO_4$ affected viability in control and *E. gracilis*

sample vials from both studies. In both cases, viability levels were > 90% with little variation between replicate vials (5.3.3). Ishikawa *et al.* (1993) has shown that *E. gracilis* requires iron for growth and in iron-deficient cells, growth is decreased to 66% in comparison with that of iron-sufficient and iron-excess (30-fold) cells. Therefore, it is highly unlikely that excess iron can adversely affect growth rates and hence viability of *E. gracilis* cells, and this is reflected in the iron viability data collected from this study. Furthermore, *E. gracilis* cells are sensitive to high saline conditions (upwards of 200 mM NaCl) and cells growing in these conditions exhibit decreases in growth rates and rates of photosynthesis (Gonzalez-Moreno *et al.*, 1997). Although the maximum amount of NaCl (5000 ppm) added to the culture medium in this study was 2 fold less than conditions created by Gonzalez-Moreno *et al.* (1997) it does show that the study conditions do not cause a decrease in algal viability levels.

5.4.3 Total antioxidant activity

5.4.3.1 Iron stress

Total antioxidant activities from *E. gracilis* samples pre-treated with various iron concentrations have shown significant differences between both controls and sample vials with the exception of samples pre-treated with 6% (w/v) $FeSO_4$ ($F_{5,29} = 38.82$; $p < 0.001$); ($F_{5,29} = 48.88$; $p < 0.001$) (Figure 5.4). The decrease in antioxidant activity for *E. gracilis* samples pre-treated with 6% (w/v) $FeSO_4$ may be due to Fe sequestration into cells. 6% (w/v) $FeSO_4$ may be a critical point in the alga, whereby cells must modify Fe regulation into cells and as a result more free iron participates in Fenton chemistry thus resulting in decreased antioxidant activity. However, at 8% and 10% (w/v) $FeSO_4$ the cells have modified Fe uptake resulting in decreased free iron and increased antioxidant activity. Substantial increases in total antioxidant activity were

detected in *E. gracilis* samples pre-treated with 2%, 4%, 8% and 10% (w/v) $FeSO_4$. This result indicates that although the iron conditions exposed to *E. gracilis* were obviously not detrimental to the algae (Figure 5.3) it did produce an antioxidant response from all samples. In this study, it was hypothesised that exposing the alga to excess iron would initiate an antioxidant response due to the possible formation of hydroxyl radicals from Fenton chemistry, which in turn react with DMSO to produce methane, but this was not the case. The substantial increase in antioxidants is likely to be due to efficient antioxidant scavenging of H_2O_2 by *E. gracilis* cells, thus limiting the amount of ·OH radicals that can react with DMSO to produce methane.

Research by Ishikawa *et al.* (1993) reported that *E. gracilis* regulate their cellular iron levels by incorporating bound iron (Fe-proteins) into their cells and in doing so suppress oxidative stress by preventing the accumulation of free cellular iron from participating in the Fenton reaction. Furthermore, *E. gracilis* cells deficient in iron lack the enzyme ascorbate peroxidase (Ishikawa *et al.*, 1993). Ascorbate peroxidase is widely distributed in iron-sufficient *E. gracilis* (Shigeoka *et al.*, 1980), higher plants (Asada, 1992) and green algae (Kow *et al.*, 1982) and functions to eliminate toxic hydrogen peroxide (Ishikawa *et al.*, 1993). *E. gracilis* cells sufficient in iron and containing ascorbate peroxidase will scavenge hydrogen peroxide, limiting the available ·OH radicals that can react with DMSO and therefore reduce methane levels. In the case of this study, the controlled uptake of cellular iron and activity of ascorbate peroxidase (and activity of SOD and peroxidase enzymes), limit the production of hydroxyl radicals in *E. gracilis* and hence methane generation in iron treated samples. Therefore it is suspected that the increase in total antioxidant activity coupled with increasing iron concentration in *E. gracilis* samples is likely to be due to enhanced ascorbate peroxidase, SOD and peroxidase activity.

5.4.3.2 Salt stress

Exposing *E. gracilis* to increasing saline conditions produces significantly different antioxidant profiles to that of control samples with the exception of control 3 and *E. gracilis* samples pre-treated with 10ppm NaCl ($F_{5,14}$ = 57.15; $p < 0.001$). Control samples have significantly lower total antioxidant activities compared to *E. gracilis* samples exposed to 100ppm – 5000ppm NaCl ($F_{5,14}$ = 22.18; $p < 0.001$) (Figure 5.4). As previously reported, exposing *E. gracilis* to 200mM NaCl decreases the rate of growth and photosynthesis (Gonzalez-Moreno *et al.*, 1997). In addition, exposing plant seedlings to excess salt conditions induces oxidative stress and increases the formation of hydrogen peroxide and hydroxyl radicals (Meneguzzo *et al.*, 1999). From the results, exposing *E. gracilis* to 10ppm NaCl produced an antioxidant response similar to that of a control sample lacking NaCl exposure. However, subjecting *E. gracilis* to a 10-fold or more increase in NaCl concentrations, does initiate an antioxidant response above control levels indicating that *E. gracilis* is sensitive to excess NaCl but it is not detrimental to the alga as reflected in the viability results. It is likely that an increase in antioxidant activity from pre-treated *E. gracilis* is due to elevated hydroxyl radical and hydrogen peroxide production, similar to the response from plant seedlings exposed to excess NaCl (Singha & Choudhuri, 1990). However, it must be noted that although the antioxidant results indicate enhanced radical production, very little methane was produced from pre-treated *E. gracilis* samples. This indicates that the antioxidant enzymes were efficient at scavenging the build-up of toxic ROS and in turn reduced the available hydroxyl radicals that could react with DMSO.

5.4.4 Comparison of *in vitro* and *in vivo* algal responses

Results presented in Chapters 3 and 4 indicate that *C. glomerata* responded in a similar manner (elevated antioxidant activity) to the test organism *E. gracilis* when exposed to biotic and abiotic stress (including salt and iron excess). Furthermore, *C. glomerata* is sensitive to heavy metal pollutants (including iron) (Whitton, 1970; 1984) and from quadrat studies (Chapter 2), it has been identified that *C. glomerata* abundance has declined in the ponds. This could be due to an increase in iron content greater than the concentrations tested in this study, which may overwhelm the *C. glomerata* antioxidant system (Fenton reaction) and subsequently cause a decline in viability and abundance. There is no evidence to suggest that *C. glomerata* is sensitive to high saline conditions, unlike *E. gracilis* (Gonzalez-Moreno *et al.*, 1997), as *C. glomerata* is relatively abundant in the ponds at saline concentrations similar to those tested in this study. However, if the concentrations of both salt and iron increase, then this may inadvertently overwhelm the antioxidant system and consequently cause a decline in *C. glomerata* viability and abundance.

5.4.5 *E. gracilis*– a potential organism for phytoremediation?

Biologically-based pollution treatment (bioremediation) can potentially clean up a variety of toxic materials at a lower cost than standard engineering practises. Most bioremediation protocols use naturally occurring bacteria to detoxify organic compounds and heavy metals. Although the process of bioremediation is mostly dominated by the use of bacteria, research carried out in many countries exploit the use of algae (phytoremediation) in waste ponds where algal activities contribute wholly or in combination with other microorganisms for the treatment and purification of wastewaters (Phang *et al.*, 2001, Fallowfield & Garrett, 1985). Algal activities supply

oxygen for bacterial oxidation of organic compounds, and they are responsible for nutrient (N, P) removal, heterotrophic assimilation of organic matter and can even bioconcentrate significant amounts of heavy metals in their tissues/cell walls (Becker, 1983; Whitton, 1984). Results from this chapter suggest that the microalga *E. gracilis* has the potential to be used an organism in phytoremediation of wastewaters due to its apparent tolerance to high iron conditions (heavy metal pollutant). The use of this organism in remediation programmes can have a significant impact on water quality by removing the heavy metal content of effluent discharges and thus reduce pollution in the aquatic environment. The concentration of iron used in this study ranged from 2% to 10% (w/v) $FeSO_4$, which are high and chosen due to their ability to drive Fenton chemistry and at these concentrations, proved non-toxic to *E. gracilis*. Therefore, it may be possible that this organism can tolerate effluent iron concentrations >10% (w/v), which can have a significant impact for promoting the use of algal organisms in future phytoremediation programmes. However, due to the sensitivity of *E. gracilis* to saline concentrations > 200 mM (~ 11,000ppm) (Meneguzzo *et al.*, 1999), salt concentration in the treatment plant would have to be carefully managed or controlled.

5.5 Conclusions

From this investigation it can be concluded that measuring ·OH radical production from *E. gracilis* using DMSO/methane evolving ·OH radical probe system proved to be a simple and sensitive technique. Methane was not detected in many *E. gracilis* samples pre-treated with salt/iron and is most likely due to the antioxidants ascorbate peroxidase and other antioxidant enzymes present in *E. gracilis* scavenging hydrogen peroxide and hydroxyl radicals before they react with DMSO to emit methane.

Viability levels for all *E. gracilis* samples pre-treated with either NaCl or iron were > 90% for all samples, thus indicating that both iron and salt are not detrimental or toxic to the alga, and in the case of iron is a prerequisite for growth and ascorbate peroxidase synthesis.

Results presented in Chapters 3 and 4 indicate that *C. glomerata* responded in a similar manner (elevated antioxidant activity) to the test organism *E. gracilis* (Chapter 5) when exposed to biotic and abiotic stress (including salt and iron excess). However, if the concentrations of salt or iron increase, then this may inadvertently overwhelm the antioxidant system and consequently cause a decline in *C. glomerata* viability and abundance.

In addition, the microalga *E. gracilis* has the potential to be used as algal organism in the phytoremediation of wastewaters due to its apparent tolerance to high iron and moderately saline conditions. This can have a significant impact for promoting the use of algal organisms in future phytoremediation programmes.

Chapter 6 GENERAL DISCUSSION

6.1 Biological functionality of SUDS ponds

This research project was designed to acquire a greater understanding of the mechanisms by which algae respond to stress (biotic and abiotic) at a physiological and biochemical level, with a view to developing criteria for assessing the biological functionality and health of SUDS ponds. The long-term objective was to highlight the potential for using algae as an indicator organism for aquatic ecosystem health and change in SUDS ponds and as a phytoremediator in urban aquatic environments. Furthermore, knowledge gained from this project may assist decision making in the practical management of SUDS operations, thus helping to enrich pond biodiversity and improve biological sustainability within the urban landscape. This discussion will provide an integrated overview of the findings presented in the thesis and will focus on the increased understanding of algal functionality in aquatic ecosystems with respect to stress (particularly xenobiotics) and highlight the importance of algae as an indicator and potential phytoremediator. It will also recommend improved pond management strategies to maximise the biological sustainability of SUDS ponds in the urban landscape.

6.2 Developing criteria to assess the biological functionality of SUDS ponds

In order to improve the current status of aquatic monitoring programmes, protocols were developed by this project to assess the biological functionality of SUDS ponds. The novel investigative techniques were designed to facilitate sampling and to predict aquatic "health" status using pond water and sediment quality, together with algal physiological and biochemical data. The objective was to enable improved pond

management regimes to be created and thus be applied to enhance the overall biological sustainability of SUDS ponds. A novel component of this project combined the use of both fundamental (oxidative stress) and applied (pond ecology) research strategies targeted at gaining a holistic understanding of algae in the context of urban aquatic ecosystem function. A model for this investigative framework is outlined in Figure 6.1.

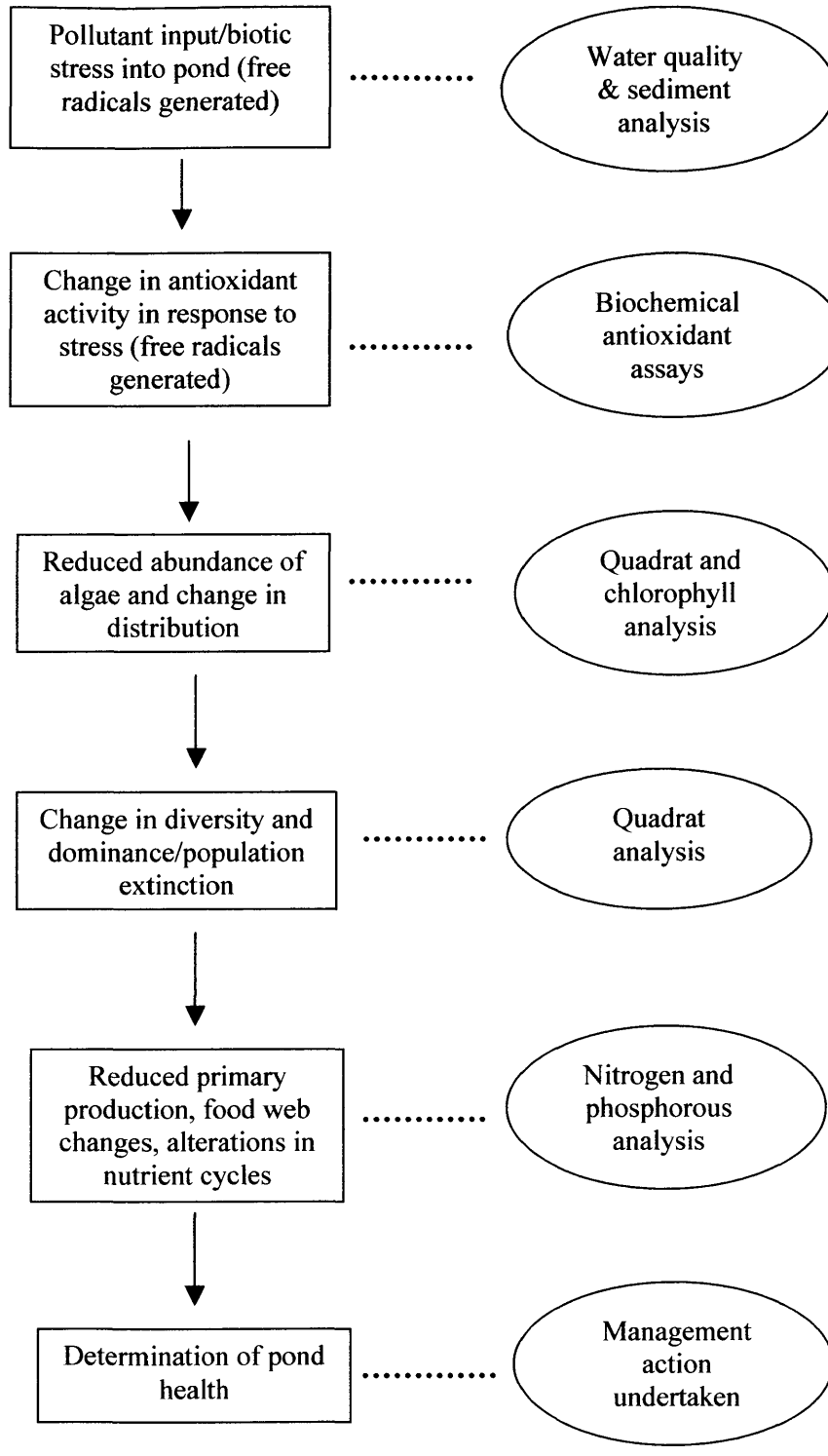


Figure 6.1 Techniques developed to study the effects of stress on *C. glomerata* from urban ponds

6.2.1 An environmental assessment of pond health

In this study the environmental assessment of pond health undertaken identified that planktonic microalgal populations within the ponds were extremely low. This may have been due to suspended solid inputs from soil erosion and construction site runoff. However, inputs of suspended solids into the ponds were generally episodic and related to high rainfall (Chapter 2) (Maitland, 1990). The low microalgal populations appears to have been due to a combination of short pond water retention times (influenced by engineering design criteria) and insufficient residual microalgal populations, which can act as an inoculum, from previous years (Chapter 2) (Talling, 1999; Reynolds & Irish, 2000). Despite the absence of blue-green blooms during the spring/summer season, which may be viewed as a success for pond design engineers, the absence of a planktonic microalgal population can have a significant impact on primary production in a waterbody and undoubtedly affect the food web balance of the pond ecosystem (Figure 6.2). However, the ponds studied had a sufficiently large macroalgal population, during the three-year sampling period, to stabilise the pond ecosystem in terms of primary production inputs. Throughout the spring/summer sampling period, it was noted from general observations that there was a significant increase in biodiversity evidenced as insect and midge populations inhabiting the ponds and its fringes, combined with breeding pairs of swans, coots and ducks (Chapter 2). The presence of such a diverse range of insects and bird species may be viewed as a sign that the ponds could sustain sufficient food web interactions to maintain a diverse aquatic environment (Figure 6.2). The dominance of *Cladophora glomerata* in all three ponds indicated that this species is highly adapted to a pond environment consisting of nutrient rich water with vegetation for it to become attached and relatively low metal concentrations in the sediments (Appendix 4B – 4D) (Figure 6.1). However, the decline in *C. glomerata*

abundance from 2001 to 2002 suggests that, although this alga was still dominant during this period, it is susceptible to changes in the pond's environmental conditions (Figure 6.1). General observations (see Chapter 2) and sediment analyses (see Appendix 4) during this period identified a significant increase in the growth of duckweed and *Phragmites australis,* which compete with algae for light, space and nutrients. Furthermore, an increase in the accumulation of heavy metal pollutants in the sediments was observed (Appendix 4B – 4D). These factors can have a significant impact on *C. glomerata* growth and reproductive success within the ponds (Figure 6.1). The decline of *C. glomerata* may thus enable new species to successfully colonise the ponds, which are more suited to the changing conditions (Figure 6.1).

The majority of contaminating pollutants entering the pond are suspended solid inputs from construction sites and as a result of soil erosion. The key "nutrient" pollutants primarily resulted from the episodic input of ammonia and nitrates from foul to storm cross-connections and the recycling of phosphorous from the sediments during reed and algal 'die-back' (2.3.4 & Appendix 4B – 4D). All of these inputs could be controlled and managed more effectively (6.6.1.4), thus reducing the damaging effects these pollutants can have on an aquatic ecosystem (Figure 6.1). An added concern is the continuous build-up of pollutants in the sediments with increasing pond age (Heal, 2002). These increases may prove problematic as they can reduce pollutant removal efficiencies, due to a decrease in pond volume and cause the resuspension of pollutants back into the water column. This process may effectively cause the decline of species susceptible to heavy metal pollution as may be the case with *C. glomerata* (Whitton, 1970; 1979; 1984) and may consequently reduce species diversity and colonization of new species within the ponds (Figure 6.1).

However, with careful management regimes, such as appropriate planting schemes, education (6.6.1) and monitoring of pollutants/sediment inputs, the ponds have the potential to sustain habitat enrichment in the longer-term, whilst performing their functional role of improving water quality and reducing flooding within urban areas.

6.3 Suitability of algae and *C. glomerata* as an indicator organism for aquatic monitoring programmes

Due to their nutritional needs and their position at the base of the food webs (Figure 6.2), algal indicators provide unique information concerning ecosystem condition compared with commonly used animal indicators. Algae respond rapidly to a wide range of pollutants at the physiological (Chapter 3) and biochemical level (Chapters 3 & 4) and thus provide a potentially useful early warning signal of deteriorating conditions and the possible causes (McCormick & Cairns, 1994). Algal assemblages provide one of the few benchmarks for establishing water quality conditions, providing a relatively unique picture of ecosystem condition and as a result diatom-based indices are routinely used by SEPA and the EA to monitor water quality, eutrophication and acidification in aquatic environments (Environment Agency, 2002). However, the problem with using algae as an indicator species is that it does not necessarily follow that the presence or absence of a single species in a specific habitat is the result of a single chemical factor (Lewis & Wang, 1997). *C. glomerata* may serve as a useful biomonitor of pond water quality as it is common in most freshwaters, tolerant to both organic and nutrient pollution, and its sensitive to heavy metal contaminants (Whitton, 1970). The presence of this alga generally indicates that the ponds are enriched with nutrients and organic pollutants, but they are not yet sufficiently polluted with metal contaminants (Whitton, 1970; Dodds & Gudder, 1992). This is reflected in the water quality data that shows the

ponds suffering from eutrophication (Chapter 2) with a steady increase in metal contamination in the sediments (Appendix 4B – 4D). The decline of *C. glomerata* abundance with increasing accumulation of heavy metals in the sediments provides further indicative evidence that this alga is sensitive to heavy metal pollutants (Chapter 2). However, it must be noted that prolonged exposure to such metals could result in decreased sensitivity, or possibly adaptation by *C. glomerata* to high metal concentrations. This might result in a false negative, whereby the presence of this alga indicates that metal pollutants are low when in fact high concentrations of metal are present in the ponds. This factor may thus limit the utility of this organism as an indicator species. Another drawback to using *Cladophora* as an indicator organism is that it is only present in the ponds for half the year and is virtually absent in late winter and early spring. The absence of microalgal populations and insufficient macroalgal growth in the ponds during these months can prove problematic for aquatic monitoring programmes using algae as indicator organisms throughout the entire year. Therefore, it may be necessary to incorporate integrative analyses of a range of biota such as invertebrate, water quality studies and diatoms (where applicable) with algae (including *Cladophora*) to fully determine the health and impact of pollutants on an aquatic ecosystem. However another approach to overcome *in situ* monitoring periodicity is the utilization of *in vitro* tests of water quality using ecotoxicity-test strains of algae (Chapter 5 & Section 6.4).

6.4 Biochemical and physiological markers of stress in *C. glomerata*

Markers of stress in the alga *C. glomerata* have been identified in this project using biochemical and physiological studies and include: the analysis of chlorophyll *a/b* ratios (Chapter 2), the development of a total antioxidant assay (Chapter 3), the determination

of specific antioxidant status (Chapter 4) and markers of free radical stress using an indirect gas chromatography technique with *E. gracilis* as the test organism (Chapter 5) (Figure 6.1).

A large proportion of the C. glomerata samples collected from Halbeath pond during June - September 2001 have lower chlorophyll *a/b* ratios than the normal ratio of 2.6:1 (Larkum & Barrett, 1983). SOD antioxidant activity during this period show enhanced levels as compared to year 2000 samples (Table 4.5 & Figure 4.2), and so indicates that exposure to high light intensity may have elevated SOD activities in response to photooxidative stress. However, it is unreasonable to assume that exposure to high light intensity is solely responsible for the SOD antioxidant response in *C. glomerata.* Enhanced levels of SOD activity have previously been identified in algal samples not only exposed to excessive light levels, but also heavy metals and herbicides (Mallick & Mohn, 2000). Significant variation in the specific antioxidant activities of *C. glomerata* collected from Halbeath pond during 2000 and 2001 has also been identified (Chapter 4). Whilst year 2000 samples exhibit enhanced glutathione reductase, glutathione-s-transferase and non-protein and protein SH group activity, the 2001 samples show a substantial decline in activity in all of the above. This result indicates that 2001 samples were not only exhibiting signs of moderate stress, but had undergone an assault by a combination of both biotic (e.g. high light intensities promote photooxidation) and abiotic factors (e.g. metal contaminants). Sediment analyses have shown that the ponds are accumulating metals in their sediments with increasing age (Appendix 4B – 4D) and the specific antioxidant enzyme (GR, GSH, non-protein and protein SH groups) data correlates with *C. glomerata* metal sensitivity (Chapter 4), whereby the decline in this enzyme group is indicative of a xenobiotic attack (heavy metals). Organic pollutants were not monitored in this study, but it is also possible that they will make a

considerable impact on overall xenobiotic loading e.g. through oil runoff from roads. However, when comparing protein bound SH group activities with protein data from Halbeath pond during 2000 and 2001, an increase in protein bound SH activity with a concomitant decrease in protein content for 2000 samples and vice versa for 2001 samples has been identified (Chapter 3 & 4). This indicates that not all of the protein in algal cells is bound to SH groups. The negligible levels of protein SH groups in 2001 samples, indicate that the sulphydryl groups were possibly oxidised and levels exhausted due to considerable oxidative damage targeting membranes (Chevrier *et al.*, 1988). However, it is hypothesised that this process may have in turn protected the rest of the algal cells therefore maintaining cell protein content and membrane integrity. In addition, enhanced protein and non-protein SH groups in 2000 samples suggests that the algal cells were again responding to increased oxidative stress and it is likely that protein content in the samples was not compromised as a result of the stress. Therefore, it is suggested that the decline in protein content during this year is probably due to life cycle stages (i.e. reproduction and senescence) within the algal samples. Furthermore, whilst significant differences were detected in specific enzyme activities between 2000 and 2001, it must be noted that no significant differences were detected in the total antioxidant activities between these years when analysed using the total antioxidant assay (Chapter 3). It may therefore be assumed that whilst the total antioxidant assay is useful initially in a monitoring programme to rapidly screen total antioxidant status in a large number of samples and highlight particular samples of interest, results from this study indicates that the assay may lack enzyme specificity and sensitivity.

By using Halbeath pond as a framework for both Linburn pond and Pond 7, it can be hypothesised that *C. glomerata* collected from these ponds could be responding to oxidative stress in a similar manner to that of Halbeath pond algal samples. Enhanced

SOD and negligible SOD was detected in a number of Linburn pond and Pond 7 samples respectively (Chapter 4). In the case of Linburn pond, this indicates that algal stability was compromised due to abiotic and biotic factors such as photooxidation at high light intensities and heavy metal damage (Mallick & Mohn, 2000). Negligible detectable SOD suggests that either Pond 7 algae were undergoing normal metabolic process such as photosynthesis, or respiration, or as with Linburn pond, they were exposed to oxidative stress. It is not possible to link increased SOD/trace SOD levels with high light intensity, as chlorophyll analyses were not performed on these samples. However, sediment analyses performed during this period indicate that heavy metals were present in the ponds (Appendix 4B – 4D), therefore suggesting that *C. glomerata* may be responding to oxidative stress. Enhanced glutathione reductase with trace levels of glutathione-s-transferase from both pond samples provides further evidence that the alga may be responsive to xenobiotic stress at the biochemical level.

Algal samples from both ponds showed significant ($p < 0.05$) differences in total antioxidant activities between months (Chapter 3) and this may be due to fluctuations in specific enzyme activities. However this result is unequivocal, as the total antioxidant assay did not identify differences in total activity between Halbeath pond 2000 and 2001 samples; these clearly had vast variations in specific enzyme activity between the two years. Protein content in both Linburn pond and Pond 7 *C. glomerata* samples varied between months, but this variation is likely to be due to life cycle stages and light intensity, as was the case with Halbeath pond samples, rather than as a result of oxidative stress.

By simulating the abiotic challenges detected in the ponds *in vitro* (excess salt and iron) using antioxidant (total antioxidant assay) and viability assessments together with

hydroxyl radical production as markers of oxidative stress in the alga *E. gracilis*, it may be possible to correlate the findings from this study with the potential of using algae in ponds as indicators of xenobiotic stress and as phytoremediators. Moreover, due to the seasonal periodicity of the presence of algae in SUDS ponds it may be useful to have an *in vitro* biotic test that can ascertain the impacts of SUDS water quality on an algal indicator species at the biochemical level (e.g. viability, total antioxidant and $^{\cdot}OH$ status monitoring). *E. gracilis* viability was unaffected (viability > 90%) by salt and iron concentration ranges selected; total antioxidant activity increased with increasing salt and iron concentrations and samples lacked methane when analysed by gas chromatography (Chapter 5). These data indicate that *E. gracilis* contains an efficient antioxidant system, thus reducing free radical mediated oxidative stress, which can cause cell damage and react with DMSO to produce methane. Furthermore, *E. gracilis* can regulate its cellular iron content (Ishikawa *et al.*, 1993) and so prevent free iron from participating in the Fenton reaction. These factors may thus enable the survival of *E. gracilis* in high iron and moderately saline conditions. From this study, it may be useful in the future to explore the possibility that *C. glomerata* sensitivity to the heavy metal iron may be related to its inability to regulate cellular iron levels, thus permitting free iron to partake in the Fenton reaction. This can eventually lead to an exhausted antioxidant system and consequently the survival and viability of *C. glomerata* may be effected. However, the total antioxidant and specific antioxidant activity data of *C. glomerata* collected from the ponds (Chapter 3 & 4) indicated that despite *C. glomerata* sensitivity to heavy metals (Whitton, 1979), this organism can survive at the salt and iron concentrations simulated in this study by enhancing its antioxidant activity. However, it has been identified through quadrat studies (Chapter 2) that *C. glomerata* abundance has declined in the ponds and may be due to an increase in iron content

similar to the upper iron concentration range tested in the *in vitro* study. This may overwhelm the *C. glomerata* antioxidant system and subsequently affect its reproductive success and cause a decline in viability and abundance (Bartosz, 1997). There is no evidence to suggest that *C. glomerata* is sensitive to high salt concentrations, unlike *E. gracilis* (Gonzalez-Moreno *et al.*, 1997), but its presence in the ponds at the concentrations selected for this study indicates that its viability and survival is unaffected. In order to fully understand the oxidative response of *C. glomerata* exposed to high salt and iron concentrations similar to those detected in the ponds, it may be necessary to undertake the *in vitro* assay as described above, and in Chapter 5, using *C. glomerata* as the test organism. However, this does pose technical problems in the evaluation of responses in macroalgae. Moreover, the ability of *E. gracilis* to tolerate relatively high concentrations of iron [10% (w/v)] highlights the possibility of using this organism in phytoremediation programmes to 'clean' iron-polluted effluents.

6.5 Algae as potential phytoremediators

The use of plant communities to stabilise, reduce or detoxify pollutants (phytoremediation) has been used worldwide in wetlands, watershed stabilization and waste treatment systems. The common reed *Phragmites australis* is one of the primary plant species used in constructed wetlands and reed bed waste treatment systems to phytoremediate effluent contaminants (Campbell & Ogden, 1999). The potential of algae as phytoremediators has been overlooked in SUDS despite pilot scale studies utilising algae to successfully phytoremediate sago starch, rubber and heavy metals from wastewater effluents and pig slurry (Phang *et al.*, 2000; 2001; Becker, 1983; Fallowfield & Garrett, 1985). Furthermore, the harvested algae can be sold off as high quality animal feed (aquaculture) or as a source of useful biochemicals (Phang *et al.*,

2000). The use of algae as the main phytoremediation organism in constructed wetlands could however prove problematic as large quantities of algae could disrupt the balance of the wetland ecosystem and yet insufficient amounts could interfere with pollutant uptake rates. It may therefore be necessary to acquire a balance between the population sizes of reeds and algae in wetlands, similar to a SUDS system. For large-scale phytoremediation programmes such as High Rate Algal Ponds (HRAP) it may be necessary to compare the removal efficiencies of algae for selected industrial effluents with plant removal rates (reed beds) and also consider the cost of implementing an operating an algal system.

6.6 Holistic approaches to enhanced pond biodiversity and sustainability

As discussed previously in Chapter 2, traditional solutions to urban drainage and water management are now viewed as unsustainable practices due to their energy use, pollution and displacement of water downstream (2.1). In order to explore the sustainable approaches to urban drainage (SUDS), a clear understanding of the definition of sustainability is required. It is generally acknowledged that sustainability encompasses three main factors, namely economic (cost and maintenance), social (interaction with people) and environmental (e.g. improved water quality and reduced flooding). Since SUDS have been implemented in the urban landscape, engineers and developers whilst focussing on the three sustainability areas have limited their consideration of long-term biological sustainability. Consequently, the full potential of these ponds as dynamic and diverse ecosystems whilst performing a functional role is not presently fully recognised by design engineers and it is in this area where SUDS ponds fall short of achieving maximal biological sustainability and hence maximal biodiversity. This project therefore combined sediment data (Appendix 4) with the

biological assessments of SUDS ponds (Chapter 2) to help understand their biological interactions and to recommend improved pond management strategies for maximising the biological sustainability of SUDS ponds in the urban landscape.

6.6.1 Problems and recommendations for pond management

6.6.1.1 Planting schemes

A potentially serious problem affecting ecosystem balance, which was highlighted by the pond survey surveys (Chapter 2), was the planting of undesirable and inappropriate plants, and particularly alien species, in the SUDS ponds. The accidental transfer, or deliberate introduction of alien and non-native plant species such as the highly invasive New Zealand pigmyweed (*Crassula helmsii*) poses a serious threat to freshwater ecosystems (Williams *et al.*, 1999). The danger of using these plants in SUDS ponds is that *Crassula* may invade Scotland's natural aquatic ecosystems that are unable to support their own indigenous native flora and fauna. This would allow *Crassula* to colonise semi-natural ponds, lakes or wetlands, therefore increasing the prevalence of this non-native plant in the landscape. The incorporation of a variety of *Phragmites australis* (from Dumfries and Galloway) to the ponds for safety reasons and pollutant uptake (phytoremediation) ensured that this plant became established in the ponds very quickly, therefore maintaining pollutant function whilst providing a habitat for native invertebrates. However, it may not be necessary to introduce a large number of plants to the ponds once they are first constructed, other than to perform a functional and safety role (*Phragmites australis*). Research shows that new ponds will colonise quickly with invertebrates and birds species and annual plants native to the area will become established after the first year (Williams *et al.*, 1999; Biggs *et al.*, 1995). The establishment of native plants to the ponds can provide the opportunity to enhance

biodiversity (plant, invertebrate, birds, animals) and the gene pool of the area without compromising pond function and safety as a priority. This consideration of native plantings should be a priority in future SUDS developments, as this will ensure natural biodiversity enrichment and biological sustainability.

6.6.1.2 Physical removal of algae from the ponds

One of the maintenance strategies carried out by the developer is to physically remove algae from the ponds at periodic intervals in order to improve pond aesthetics for local residents. However, episodic removal of large alga from the ponds poses a serious threat to food web ecosystem dynamics and invertebrate habitats (Figure 6.2). This can cause a major problem in primary food web function. Algal taxa vary greatly in their edibility and shifts in species composition can affect feeding relationships, population growth and structure at higher trophic levels in aquatic food webs (Porter, 1976). Removal of algae will also influence the dynamics of primary production (photosynthesis), which will in turn affect water quality parameters important to biota (pH, nutrients, O_2, CO_2) as shown in Figure 6.2. Not only can algal removal allow the invasive duckweed to grow, blocking out light for submerged plants and invertebrates, but also the area in which *C. glomerata* alga colonised was one of the shallowest and richest parts of the pond (2 - 5 cm) (Pond Action, 2000). Due to the ecological importance of shallow waters to pond invertebrates, great care is required to ensure that management regimes do not destroy invertebrate habitats or food supplies along the pond margins. Furthermore, data collected during this period (Chapter 2) shows that there was no justification to removing this alga other than to improve pond aesthetics.

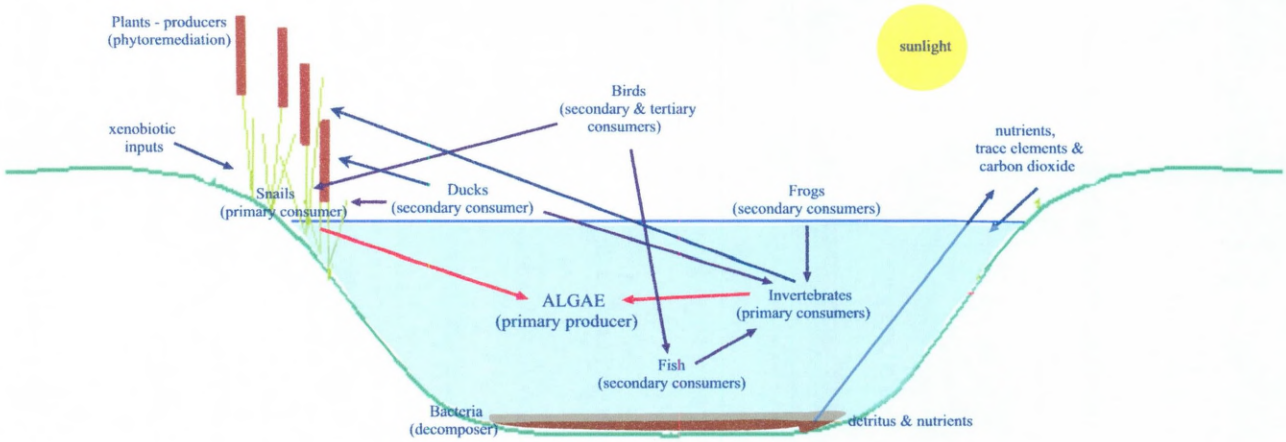


Figure 6.2 Diagram of pond food web interactions showing the pivotal role of algae in aquatic systems

6.6.1.3 Education and communication with local residents

In order to maintain efficient pond function and ecosystem balance it is necessary to educate and communicate with local residents in areas where SUDS are to be implemented. This should be undertaken in advance of the building of SUDS facilities. Residents ideally may be educated on the negative impacts that feeding birds, alien introductions (fish tank plants) and exotic fish can have on pond function, ecosystem balance and biological sustainability. Native birds such as swans in the ponds are aesthetically pleasing to locals and if numbers are controlled they can help to maintain food web and ecosystem balance (Figure 6.2). However, problems can occur when residents feed the birds. This can cause an increase in bird populations from the surrounding area, resulting in enhanced faecal matter inputs and nutrients from foodstuffs (e.g. bread, grain) which may influence pond eutrophication, as well as damage to plants and pond margins (Williams *et al.*, 1999). This can result in a severe reduction of pond invertebrates and consequently a decline in native species diversity. In addition, the ponds were designed to be a fish-free zone and with the introduction of perch by local residents may have inadvertently reduced the numbers and habitats of invertebrate species, which cannot tolerate the predation pressure and other impacts of fish. Moreover, their introduction may also reduce water quality due to increased faecal inputs (Williams *et al.*, 1999). It is therefore necessary to communicate with local residents before ponds are constructed to help develop a local understanding of the functional role of the ponds and to encourage responsibility and ownership in maintaining healthy pond systems (e.g. reducing litter dropping, and the scooping up of dog faeces). It may also be beneficial to link with local schools and community groups such as Scottish Natural Heritage to encourage projects/biodiversity surveys, which may

help to promote an interest within the local community of pond ecosystems, whilst providing invaluable information on pond diversity for the SUDS consortium.

6.6.1.4 Improvements to pond water quality

One of the main environmental problems with SUDS ponds is that they can create a 'sink' for pollutants and accumulate large quantities of toxic metals in the sediments. These polluted sediments must be removed from the pond by dredging in order to maintain adequate pond function (Williams *et al.*, 1999; Biggs *et al.*, 1995). However, this practice is not only unsustainable (polluted sediments transferred to landfill) but will invariably disrupt the balance of the pond ecosystem. Suspended solids inputs can be controlled or managed more effectively by adding vegetated silt traps or sediment traps to retain the sediment before it enters the pond thus reducing the amount of sediment and pollutants entering the pond. The application of sediment traps to ponds may not only prolong the life of a pond (reducing the need to dredge every 10 – 15 years), but also remove a large proportion of pollutants, which may impact the pond ecosystem. The sediment traps could then be dealt with using a reed bed phytoremediation system to reduce the toxicity of the pollutants through active uptake. This practice is being undertaken by Tayside Contracts in Forfar to treat waste recovered from road gullies (Tayside Contracts, 2003).

The phosphate and nitrate levels of the ponds could be improved by reducing the incidence of foul inputs into the ponds from foul to storm cross-connections, and by ensuring developers correctly identify and connect into foul sewers instead of surface water pipes. Controlling bird populations and their foodstuffs (e.g. bread, grain) should also help reduce nutrient inputs and thus limit excessive algal growth. To prevent large

quantities of phosphate being recycled back into the pond during reed 'die-back' in late autumn, a proportion of dying *Phragmites australis* should be removed from the ponds. This could reduce the available phosphate in the ponds and prevent eutrophication. This action should not interfere with pond function or ecological balance as new *Phragmites* regenerate.

It is important however, to note that whilst adequate pond management is an essential consideration for SUDS ponds, it is necessary to strike a balance between a managed and an unmanaged pond. Achieving this balance could result in a 'natural' pond system within engineering and landscaping constraints whilst ensuring hydrological functionality and biological sustainability.

6.7 Action steps for improved pond management strategies

DO NOT:

- Plant inappropriate and non-native plant species;
- Introduce a large number of plants once constructed other than to maintain pond function and safety;
- Remove algae from the ponds unless they are posing a serious health threat;
- Physically disrupt the pond margins (2 – 5cm water depth);
- Introduce alien and exotic fish species.

DO:

- Educate and communicate with local residents before SUDS are implemented;
- Encourage biodiversity projects/surveys with local environment groups and schools;
- Apply sediment/vegetated silt traps to the pond inlets;
- Remove silt/sediments from the traps and apply to reed beds;
- Reduce the incidence of foul inputs to the ponds;
- Control pond bird populations;

- Remove a proportion of dying *Phragmites australis* from the ponds during autumn
- Monitor biological components of ponds as well as water quality and hydrology.

6.8 Future work

6.8.1 Fundamental Research

The use of antioxidants as markers of stress in *C. glomerata* has provided a valuable insight into the biochemical response of this alga to stress (biotic and abiotic) in an urban aquatic environment (Chapter 3, 4). However, in order to obtain a greater understanding of the generic stress response in algae, it may be necessary to further investigate and develop antioxidant assays such as ascorbate and glutathione peroxidase, not only for *C. glomerata* but for other algal organisms commonly associated with urban aquatic environments and relate the antioxidant status to aquatic health. Furthermore, it may also be necessary to undertake free radical studies to fully determine that the rise/fall of antioxidants detected in *C. glomerata* exposed to stress is due to enhanced free radical production.

It would be both interesting and useful to further develop the gas chromatography assay to investigate markers of free radical activity in *C. glomerata* and other algal organisms exposed to heavy metals and other urban pollutants. This would hopefully permit a more detailed understanding of fluctuations in antioxidant status and identify species that are tolerant or sensitive to a particular contaminant. In addition, it may be useful to explore the intracellular regulatory mechanism by which *C. glomerata* incorporates iron into cells and relate this to sensitivity to iron and compare with iron tolerant algal species. Furthermore, the development of these assays (antioxidants and gas chromatography) could be particularly useful in selecting algal species, which may have

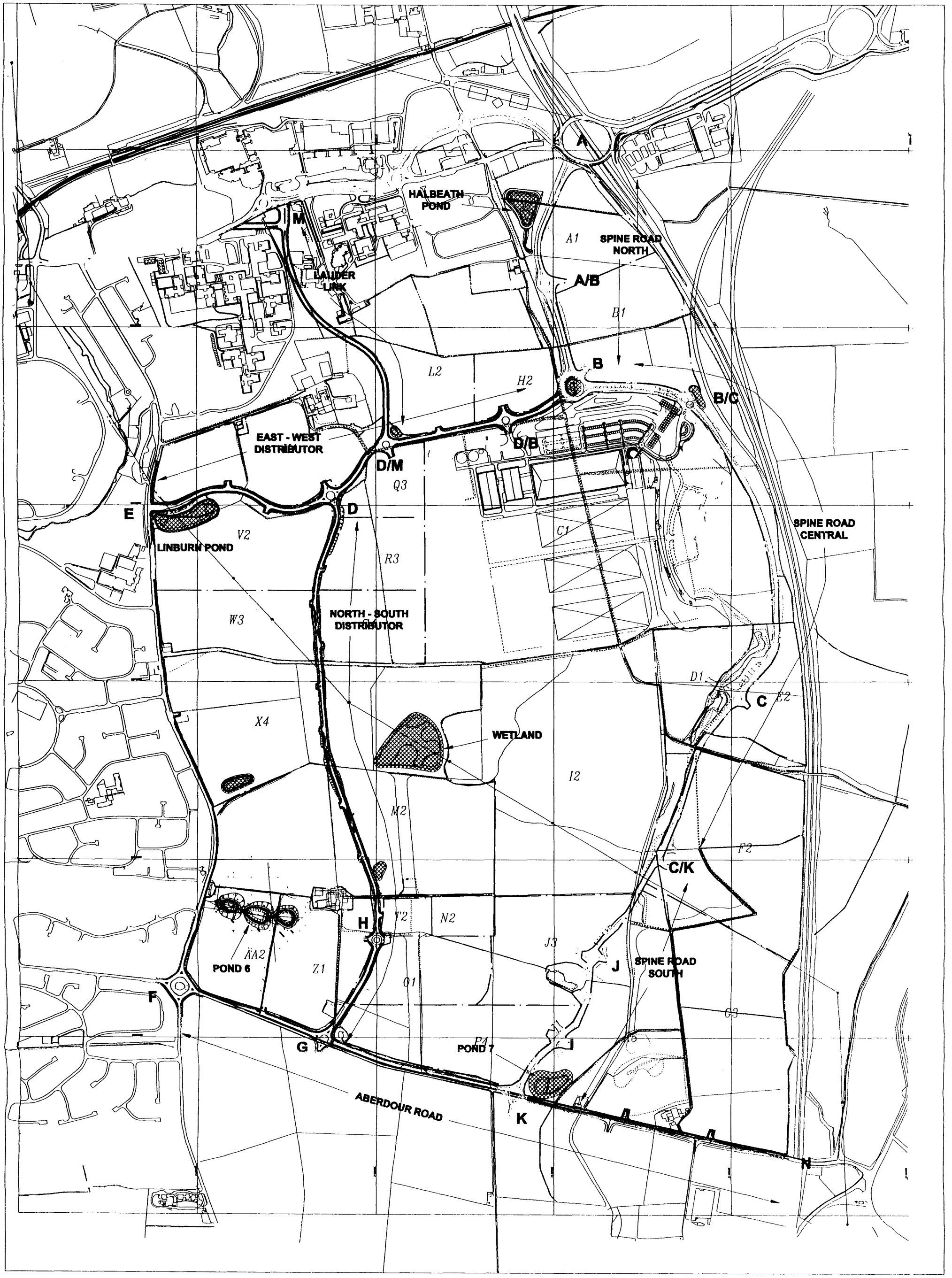
the capacity and potential to tolerate and phytoremediate contaminated effluents. *In vitro* assays once developed may also be useful in out-of-season monitoring of pond water quality.

6.8.2 Applied Research

The wider context of the project development would further benefit from implementing pond biodiversity monitoring schemes rather than focussing solely on hydrological function and sediment content. The surveys shown in Chapter 2, could be re-designed to incorporate and include relative number/genus of vertebrate, bird and plant species surveys. In addition, a River Invertebrate Prediction and Classification System (RIVPAC) analysis could be undertaken which could assess the biological quality of the SUDS ponds with respect to observed and predicted macroinvertebrate fauna (Wright, 2000). These biodiversity surveys could be undertaken by local schoolchildren as school projects or by environmental groups with expertise in plant, invertebrate, vertebrate and bird identification. This information would not only be invaluable for the SUDS consortium but could provide information regarding pond stability and health with respect to food web interactions and water quality (Figure 6.2).

Ultimately, by gaining an holistic understanding of SUDS ponds incorporating hydrological function, biological/chemical interactions, algal stress physiology responses, sediment content and biodiversity assessments, it may be possible to develop an integrative model to draw together the currently disparate data sets that have been accrued from surveys and pond studies. Such an integrated model system would have the capacity to inform engineering construction and management protocols to improve

pond design so maximising the biological sustainability of SUDS ponds in the urban landscape.



Legend

- Primary Distributor
- District Distributor
- Local Distributor

DATE	REVISION	NO	INTL
29/6/99	Linburn Road and Aberdour Road definition amended.	A	JT

PROJECT: Duloch Park

CLIENT: Wilcon Homes (Scotland) Ltd.

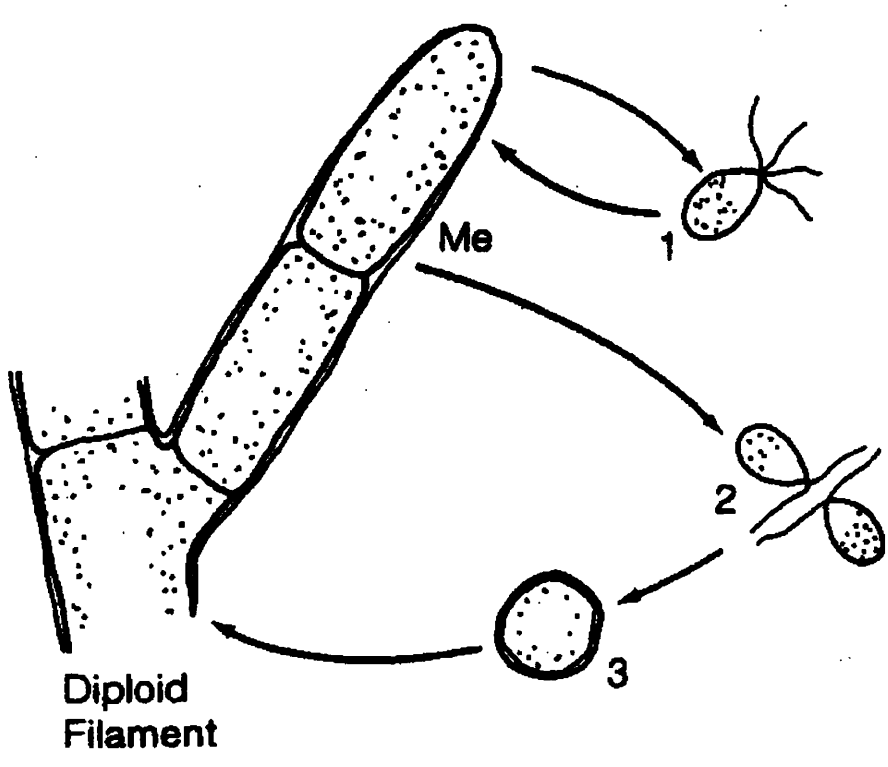
TITLE: Principal Road Network Hierarchy

IRONSIDE FARRAR
ENVIRONMENTAL CONSULTANTS

DRAWN	DRAWN	DRAWN
JT		09/03/99

SCALE	DWG NO	REV
Hz 1:10,000@A3 Vt	5815/103	A

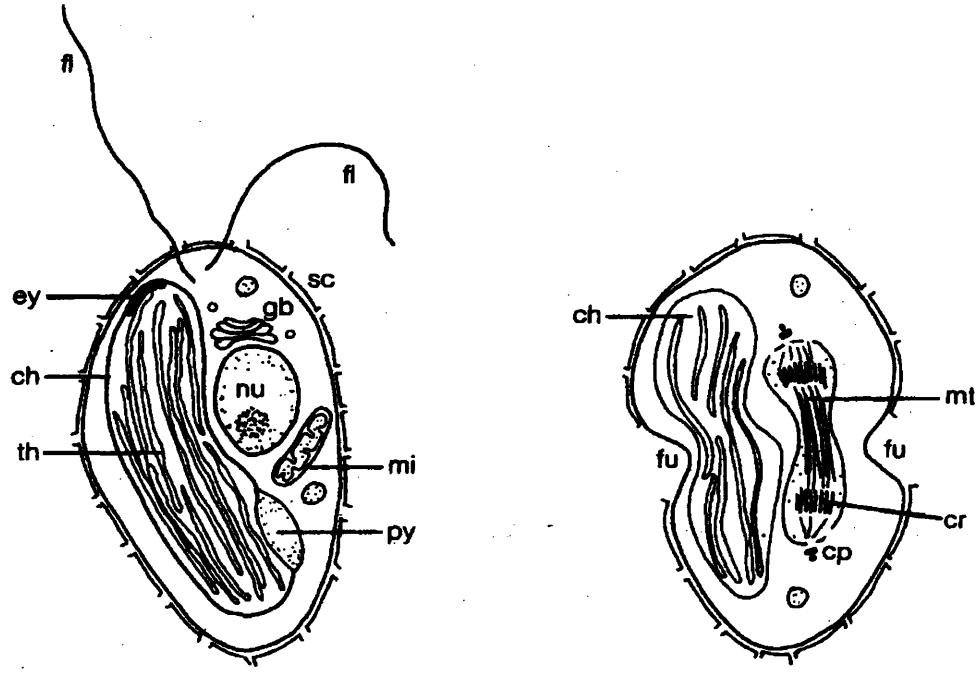
Appendix 2A **Life cycle of *Cladophora glomerata***



Life cycle of *C. glomerata* lacks a haploid phase. Filaments are diploid and reproduce either asexually forming diploid zoospores (1) or sexually forming haploid gametes (2). Gametes then fuse to form a zygote (3), resulting in a diploid thallus (Sze, 1998).

Me = meiosis in gametangium.

Appendix 2B **Life cycle of *Euglena gracilis***



Reproduction in *E. gracilis* occurs by longitudinal division of cells. Sexual reproduction is unknown in euglenophytes (Sze, 1998).

(a) Structure of a vegetated cell; (b) asexual reproduction involving mitosis and cell division by furrowing.

ch = chloroplast; cp = centriole pair; cr = chromosomes; ey = eyespot; fl = flagellum; fu = furrow; gb = golgi body; mi = mitochondrion; mt = spindle of microtubules; nu = nucleus; py = pyrenoid; sc = scale; th = thylakoid.

Appendix 3 Decision making processes and rationale development for field studies

Stage 1 - Selection of indicator organism

Preliminary surveillance of the study ponds prior to sampling (March 2000) identified macroalgae at the pond margins either as free floating mats or attached to the common reed *Phragmites australis*. During these visits, small samples of macroalgae were removed from the pond by hand for identification purposes. Upon microscopic investigations in the laboratory, it was identified that there were three species of macroalgae present in each of the study ponds. All three were green, filamentous Chlorophytes and were identified as *Oedogonium*, *Spirogyra* and *Cladophora glomerata*. Further visual inspections showed that *C. glomerata* was the most abundant alga at the pond margins compared to both *Oedogonium* and *Spirogyra*, which were present at sporadic intervals. It was therefore decided that *C. glomerata* would provide adequate biomass for sampling without causing major impact to pond dynamics and due to its ease of identification and location at the pond fringes, *C. glomerata* was chosen as the indicator organism for this project.

Stage 2 – Development of quantitative assessments

Determining microalgal biomass using standardised methods i.e. chlorophyll *a* analysis (Standing Committee of Analysts, 1983), was made problematic for this project due to a lack of sufficient microalgae present in the study ponds (2.4.1). In order to quantitatively assess the relative abundance of macroalgae within the ponds a new approach/methodology was required. Field plant ecologists commonly use quadrats to assess the frequency/abundance of plants within a specified area and due to the floating

properties (2.4.2) and large filament size of *C. glomerata* enables quadrat methodology to be adopted from plant ecology and applied to algal physiology. This technique was chosen to quantitatively assess *C. glomerata* abundance at the pond margins based on percentage algal coverage within a quadrat at specific pond areas i.e. inlets and outlets.

Stage 3 - Expert advice

Advice regarding the quadrat sampling regime was sought from a field ecologist (Dr. Bill Block) and a biological statistician (Professor Harry Staines). Dr Block agreed that quadrat methods could be harnessed from the field of plant ecology and applied to macroalgae and Professor Staines confirmed that the quadrat sampling regime used in this project would enable the quantitative assessment of *C. glomerata* abundance within the pond margins to be determined.

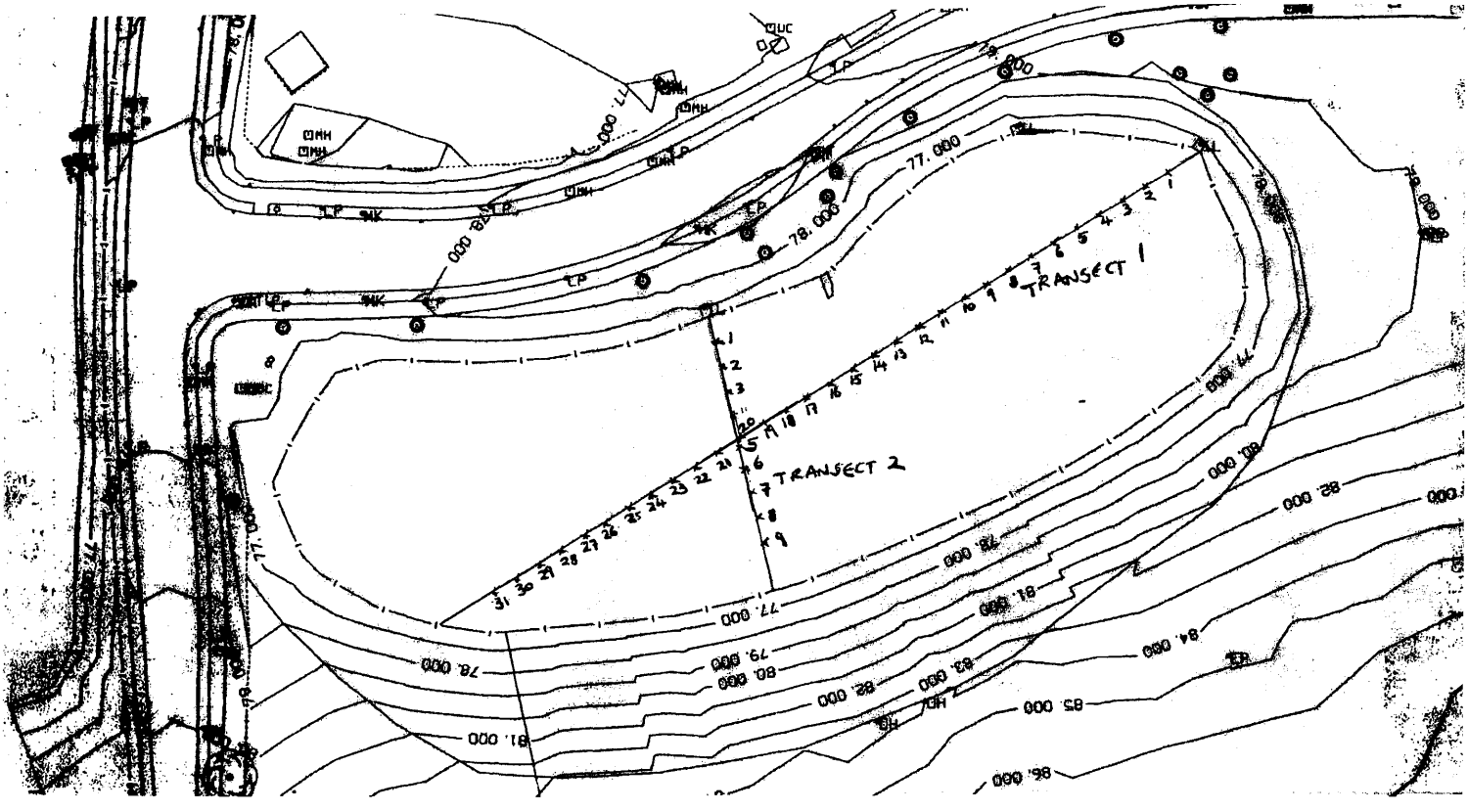
Stage 4 – Quadrat sampling in place

The rationale developed for the quantitative assessment of macroalgal biomass is largely based on practicalities such as ease of sampling at the pond margins, accessibility at pond fringes, abundance of the selected algal indicator organism and safety. These issues are addressed in the quadrat methodology (2.2.4) and the sampling strategy set in place.

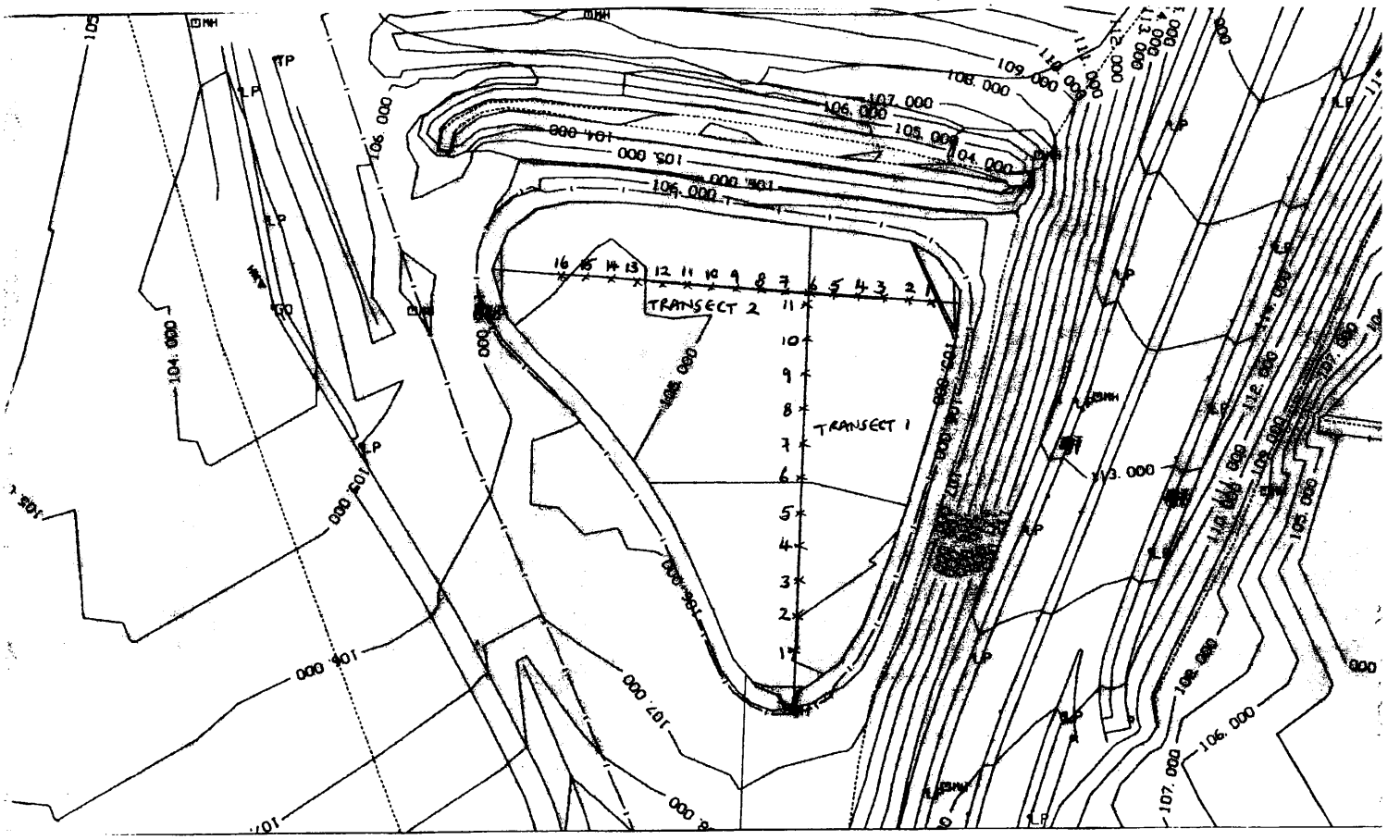
Appendix 4A Sediment Transects

Sediment samples were taken on an annual basis from 1999 to 2001 by Dr Kate Heal at a number of marked endpoints along two transects and analysed for heavy metals, nitrogen and phosphate levels (Heal, 2002).

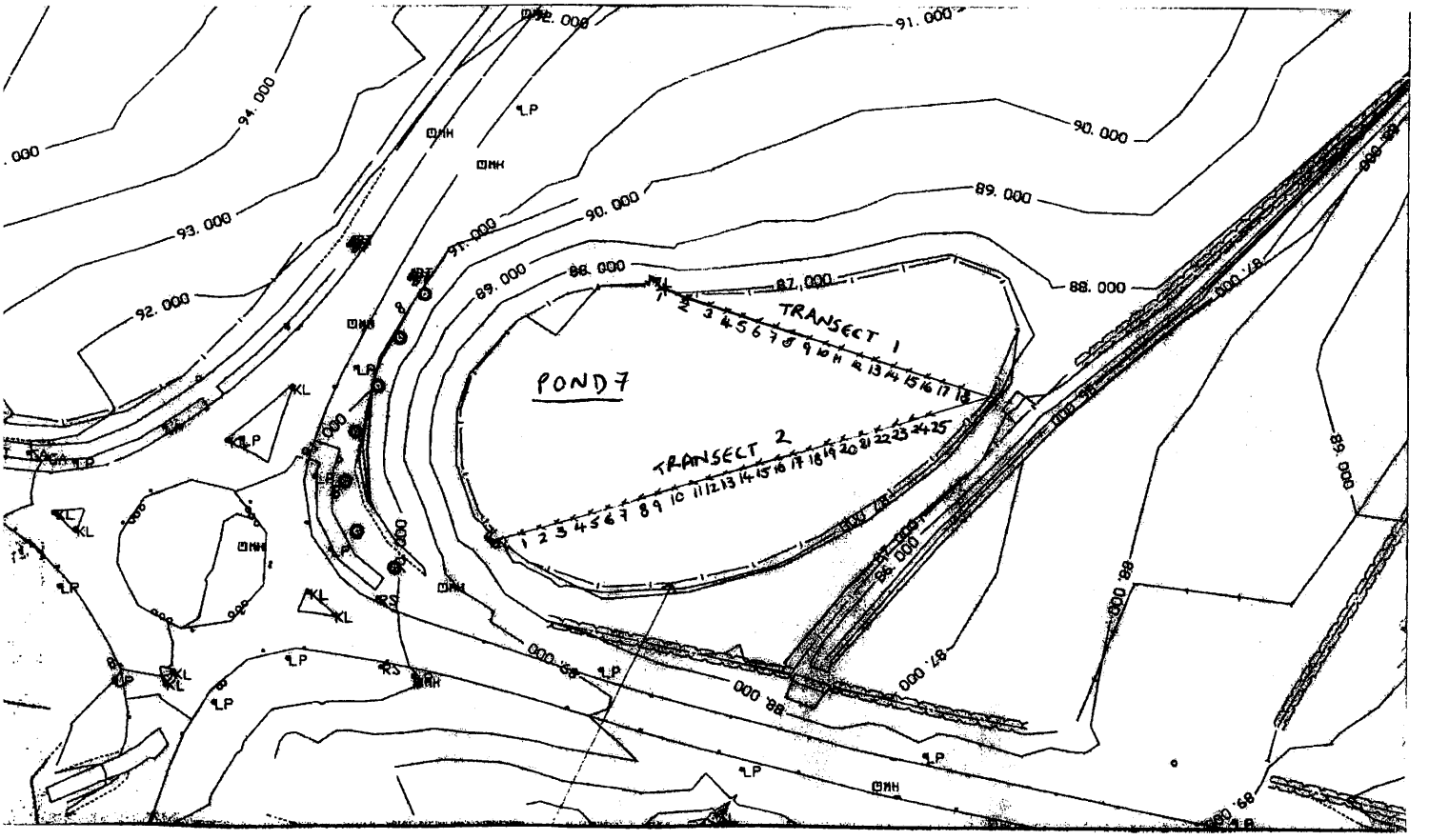
Linburn Pond (Scale 1:500)



Halbeath Pond (Scale 1:250)



Pond 7 (Scale 1:250)



Appendix 4B Sediment accumulation of metals and nutrients in 1999

Pond	Transect point	Zn $mg.kg^{-1}$	Cr $mg.kg^{-1}$	Ni $mg.kg^{-1}$	Cu $mg.kg^{-1}$	Cd $mg.kg^{-1}$	Pb $mg.kg^{-1}$	Fe $mg.kg^{-1}$	Total N $mg.kg^{-1}$	Total P $mg.kg^{-1}$
Halbeath	Inlet	67.5	29.9	33.6	22.3	0.0	11.1	51603	2396	909
Halbeath	T1.1	23.9	18.8	19.9	17.5	0.0	12.4	39687	1759	824
Halbeath	T1.2	18.2	12.9	10.8	11.9	0.0	6.6	46541	1333	535
Halbeath	T1.3	19.4	25.8	17.0	14.3	0.0	10.6	31918	1393	552
Halbeath	T2.1	15.8	7.5	17.2	14.0	0.0	8.4	39629	867	549
Halbeath	T2.2	18.6	25.3	16.7	14.8	0.1	9.4	36266	1350	587
Halbeath	T2.3	31.3	8.3	14.5	16.0	1.1	8.0	41773	977	372
Halbeath	T2.4	33.9	18.7	23.5	16.0	0.0	12.4	25914	1093	529
Linburn	East Inlet	54.0	19.2	21.6	14.5	1.6	19.7	52762	2363	695
Linburn	North 1 Inlet	73.9	14.2	11.8	19.0	0.6	9.9	32122	1092	524
Linburn	T1.1	73.1	46.5	43.6	23.3	0.0	25.2	43759	3538	2696
Linburn	T1.2	77.9	64.2	54.4	23.1	0.0	24.2	57936	3228	2004
Linburn	T1.3	79.6	70.7	49.0	24.2	0.0	24.8	42552	3374	889
Linburn	T1.4	66.6	54.8	42.1	23.3	0.0	25.4	31226	2212	804
Linburn	T1.5	78.8	67.7	57.5	23.2	0.0	29.4	46710	2361	902
Linburn	T1.6	54.2	42.2	41.8	17.7	0.0	22.2	32832	2745	898
Linburn	T2.1	62.7	70.2	46.9	18.4	0.0	17.1	31839	1820	588
Linburn	T2.2	71.9	93.6	71.1	20.9	0.0	18.9	28473	2356	745
Pond 7	East Inlet	49.0	17.5	13.3	12.0	0.3	12.8	30994	1437	575
Pond 7	T1.1	61.2	27.0	23.0	12.9	0.0	23.4	36735	1798	542
Pond 7	T1.2	59.2	19.7	29.2	17.2	0.0	8.7	23695	1795	373
Pond 7	T1.3	62.7	31.5	30.6	15.6	0.0	15.0	11831	1625	508
Pond 7	T1.4	60.4	32.2	34.0	19.3	0.0	11.8	30607	1519	470
Pond 7	T2.1	68.5	42.7	36.2	21.9	0.0	18.5	35427	3216	907
Pond 7	T2.2	80.9	38.7	33.8	18.8	0.0	22.9	36797	1980	519
Pond 7	T2.3	43.3	35.1	30.7	16.2	0.0	13.4	42724	1813	493
Pond 7	T2.4	59.3	37.8	35.5	19.0	0.0	14.0	39299	2143	556
Pond 7	T2.5	74.2	34.6	33.0	16.2	0.0	14.5	40602	1467	629

Appendix 4C Sediment accumulation of metals and nutrients in 2000

Pond	Transect point	Zn $mg.kg^{-1}$	Cr $mg.kg^{-1}$	Ni $mg.kg^{-1}$	Cu $mg.kg^{-1}$	Cd $mg.kg^{-1}$	Pb $mg.kg^{-1}$	Fe $mg.kg^{-1}$	Total N $mg.kg^{-1}$	Total P $mg.kg^{-1}$
Halbeath	Inlet	50.6	21.8	15.3	12.6	0.0	17.4	36893	434	4822
Halbeath	T1.1	115.1	44.0	60.6	14.1	0.0	20.5	58086	1441	1031
Halbeath	T1.2	79.1	43.5	38.7	12.4	0.0	21.0	60824	1492	1020
Halbeath	T1.3	41.6	17.7	23.3	9.0	0.3	10.9	44749	842	712
Halbeath	T2.1	56.1	28.3	25.4	10.2	0.0	13.1	45189	922	651
Halbeath	T2.2	47.0	18.3	23.2	10.6	0.0	12.4	48932	703	639
Halbeath	T2.3	50.8	22.5	22.8	13.9	0.0	26.5	40821	583	578
Halbeath	T2.4	46.6	14.0	25.7	9.7	0.0	10.3	44241	770	5167
Linburn	East Inlet	67.5	23.9	23.9	8.6	0.0	10.3	37990	1225	221
Linburn	North 1 Inlet	202.6	28.1	23.7	19.6	0.1	25.8	36283	1300	427
Linburn	T1.1	81.6	18.4	29.7	8.3	0.0	15.0	34339	1594	465
Linburn	T1.2	78.6	26.8	42.2	9.6	0.0	19.7	66757	1779	530
Linburn	T1.3	90.3	27.8	41.3	11.0	0.0	19.8	61359	1687	506
Linburn	T1.4	70.8	26.5	33.4	10.1	0.0	14.6	46755	1153	350
Linburn	T1.5	98.1	28.7	35.1	10.5	0.0	12.9	48125	1208	341
Linburn	T1.6	65.2	23.4	34.3	8.7	0.0	13.6	45264	1207	437
Linburn	T2.1	67.5	23.6	32.1	10.2	0.0	11.5	43783	892	330
Linburn	T2.2	58.7	22.4	31.1	9.3	0.0	10.5	31428	920	241
Pond 7	East Inlet	115.1	23.9	24.4	21.1	0.3	19.4	49879	1075	1054
Pond 7	T1.1	60.3	23.0	24.5	9.6	0.0	12.5	42907	1085	541
Pond 7	T1.2	79.9	33.8	28.2	9.9	0.1	22.4	41218	1448	878
Pond 7	T1.3	68.1	30.0	26.9	11.7	0.0	15.9	45114	1165	786
Pond 7	T1.4	64.6	26.6	26.5	11.1	0.0	14.2	39562	915	770
Pond 7	T2.1	85.9	36.8	37.2	10.9	0.0	20.4	56388	1720	1046
Pond 7	T2.2	67.3	32.4	29.1	8.4	0.0	13.8	48630	1248	795
Pond 7	T2.3	65.2	28.6	29.5	9.8	0.0	16.6	45881	1329	777
Pond 7	T2.4	62.5	28.4	28.9	10.8	0.1	12.5	48927	1094	889
Pond 7	T2.5	57.5	29.9	27.1	8.4	0.1	12.9	39624	1168	693

Appendix 4D Sediment accumulation of metals and nutrients in 2001

Pond	Transect point	Zn $mg.kg^{-1}$	Cr $mg.kg^{-1}$	Ni $mg.kg^{-1}$	Cu $mg.kg^{-1}$	Cd $mg.kg^{-1}$	Pb $mg.kg^{-1}$	Fe $mg.kg^{-1}$	Total N $mg.kg^{-1}$	Total P $mg.kg^{-1}$
Halbeath	Inlet	87.7	236.0	194.0	21.3	0.6	27.2	30179	467	232
Halbeath	T1.1	135.1	91.3	88.0	22.9	0.6	57.7	51356	1136	610
Halbeath	T1.2	53.13	148.7	106.1	16.0	0.1	25.2	45645	359	385
Halbeath	T1.3	35.0	80.6	71.3	10.5	0.1	23.1	27449	498	275
Halbeath	T2.1	53.6	104.2	94.1	14.6	0.3	17.0	34500	598	300
Halbeath	T2.2	51.5	207.8	166.0	17.0	0.0	160.6	33881	590	394
Halbeath	T2.3	51.5	181.5	165.0	16.4	0.0	153.4	35427	533	332
Halbeath	T2.4	55.2	172.7	112.3	17.2	0.1	57.4	48312	473	366
Linburn	East Inlet	238.4	377.0	183.0	46.7	0.1	117.5	32751	1145	300
Linburn	North 1 Inlet	46.1	466.0	216.1	12.9	0.2	25.9	19232	256	220
Linburn	T1.1	105.6	56.2	83.6	19.6	0.1	34.8	59474	2136	382
Linburn	T1.2	96.2	42.4	71.8	16.6	0.0	36.1	44893	2065	390
Linburn	T1.3	67.0	119.6	108.4	14.7	0.0	17.3	35690	1263	452
Linburn	T1.4	98.5	80.4	85.5	14.5	0.0	19.9	47581	1066	455
Linburn	T1.5	85.3	58.0	80.8	16.9	0.0	23.4	39474	1356	464
Linburn	T1.6	93.7	102.6	100.8	17.2	0.1	32.4	49156	1937	523
Linburn	T2.1	61.1	226.7	124.1	15.2	0.0	21.3	35377	1144	352
Linburn	T2.2	100.0	78.4	88.7	19.2	0.0	35.5	35466	1628	416
Pond 7	East Inlet	107.7	407.7	214.7	31.5	0.7	42.4	46792	646	263
Pond 7	T1.1	68.0	187.5	121.8	29.0	0.5	32.3	44047	792	365
Pond 7	T1.2	54.1	218.6	135.8	12.7	0.2	10.1	31006	512	384
Pond 7	T1.3	59.1	170.6	117.5	13.5	0.4	32.9	32079	763	376
Pond 7	T1.4	88.8	337.7	209.0	15.2	0.7	27.8	30709	566	359
Pond 7	T2.1	58.7	192.7	135.0	14.5	0.5	17.0	32075	710	338
Pond 7	T2.2	52.8	283.3	164.6	15.9	0.5	7.0	44766	508	342
Pond 7	T2.3	63.9	477.3	284.5	16.3	0.7	13.5	43446	651	356
Pond 7	T2.4	47.9	476.1	257.7	15.4	0.4	15.4	30023	406	371
Pond 7	T2.5	54.7	185.0	121.4	15.4	0.3	56.9	30752	884	444

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